OVERWINTERING PHYSIOLOGY OF ARCTIC AND SUBARCTIC INSECTS

FROM INTERIOR ALASKA

By

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A

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By

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Abstract

This dissertation focuses on the overwintering of three insects from Interior Alaska: a hemipteran, *Elasmostethus interstinctus*, and a coleopteran, *Cucujus clavipes puniceus*, that are freeze avoiding in the strict sense of the phrase, and a dipteran, *Exechia nugatoria*, that is simultaneously partially freeze avoiding and freeze tolerant. The variability within the freeze avoidance strategy itself is a key theme throughout this dissertation.

Two significant contributions to comparative physiology are the confirmation of insect vitrification (glass formation) with its attendant extension of freeze avoidance and survival into a new, extreme low temperature record of -100°C and the simultaneous coupling of freeze avoidance and tolerance within an individual, which may more properly be described as a new overwintering strategy. Vitrification is the process by which ice crystallization is circumvented, resulting in a supercooled amorphous solid. Through a combination of antifreeze proteins that inhibit ice nucleation, dehydration tolerance, presence of high glycerol concentration, and low temperatures, the mobility of the remaining liquid water molecules is reduced, effectively by-passing the crystalline state. The second contribution is the discovery of a new overwintering strategy that combines freeze avoidance and freeze tolerance within an individual. In this case, the abdomen freezes (and the insect survives), while the contiguous head/thorax remains supercooled.

These findings lead to the following evolutionary and trans-disciplinary questions. Is vitrification an adaptation? What is the selective advantage of compartmentalizing ice

between body sections of an individual insect? Is this new overwintering strategy an example of a species transitioning between either becoming exclusively freeze avoiding or free tolerant? Applying new understanding of mechanisms of insect vitrification and avoidance of devitrification to cryomedicine may extend preservation of human tissues and organs. Similarly, for physical and material scientists, by understanding the patterns of ice formation within insects that tolerate, inhibit, and/or impede ice formation below the homogeneous ice nucleation temperature of water (-40°C), new biomimetic possibilities can be envisioned.

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Dedicated to

My wife Barbara Tudor

My parents Linda and Larry

My brothers (in-law) and sister (in-laws) and their kids:

Pete and Michele–Justin and Carley

Julie and Phil–Erik and Emma

Mike and Maria–Colin, Sophia, and . . . ?

Introduction: Brief History of Research on Insect Overwintering Physiology in Alaska

What follows is a brief review of insect overwintering physiology conducted in Alaska by researchers affiliated with the University of Alaska Fairbanks (UAF) and/or the Institute of Arctic Biology (IAB). This is an abbreviated history and is not meant to be a complete review. In fact, many additional UAF/IAB biologists have also contributed to overwintering research and their work cited. For instance, two of Stephen F. MacLean's works (MacLean 1973 and MacLean and Hodkinson 1980) correlate temperature, life histories, and distributions of high latitude insects, especially on the North Slope of Alaska. These works have contributed to an understanding of overwintering based on low temperature effects; however, this introduction is intended to serve as a record of the people and papers that have more directly influenced the research in this dissertation.

General Insect Research and Climate Characteristics in Alaska

Insect research in Alaska has been conducted on and off for over 100 years. The first major report of insects in Alaska was carried out by the Harriman Alaska Expedition, from 1 June to 1 August 1899. Alaska was regarded as "terra incognita," especially for hymenopterologists who had known of only 30 species prior to the Expedition. By the end of the two month journey, 335 species were added (W. Ashmead in Harriman Alaska Expedition, 1899 (1910), Vol. IX). Overall, the collection of insects in Alaska increased by nearly 8,000 specimens representing a "thousand species" under the direction of Trevor Kincaid, entomologist of the Expedition (Merriam, Harriman

Alaska Expedition, 1899 (1910), Vol. VIII). Even today, new species continue to be found (D. Sikes, UAF/IAB, Pers. Comm.). While the Harriman Alaska Expedition focused on insects from southeast to south central Alaska (Juneau to Seldovia), a more northern focus took place in the 1960s near Point Hope Alaska. Concern over the health of Inupiaq-Eskimos was raised in relation to Project Chariot, a program sponsored by the Atomic Energy Commission, whereby an "instant harbor" was to be created by atomic detonations (initially in the megaton range) at Ogoturuk Creek near Point Hope, Alaska (O'Neill 1995). A proto-environmental impact statement that came to be known as *Environment of the Cape Thompson Region, Alaska*, was completed in 1966. The chapter in this report entitled "Terrestrial Invertebrates" includes lists of both terrestrial and freshwater invertebrates of the Ogoturuk Valley collected in the summers of 1959-1961 (Watson *et al.* 1966). Besides systematics, more recent studies on insect thermoregulation, ranging from bees (Bishop and Armbruster 1999) to dragonflies (Sformo and Doak 2006), have been completed.

One feature common to all of the above research, however, is that each was conducted during relatively warm conditions from spring through fall and not under winter-like conditions. Yet, Interior Alaska, an area co-extensive with the taiga forest and taiga snow-type (Sturm *et al.* 1995) extending roughly south of the Brooks Range (68° 02' N) to the Alaska Range (62° 58' N) (Benson 2001) may have winter-like conditions between 150 to 200 days of the year (Sturm *et al.* 1995; Benson 2001; Shulski and Wendler 2007). The climate of Interior Alaska has been classified as extreme continental climate due to its long duration of snow cover, low temperature range, and

persistent thermal gradient within the snow pack (Benson 2001). Wiseman and Fairbanks, Alaska, are two locations that have experienced some of the coldest environments in North America. In 1971, for instance, the Prospect Creek Camp, 235 km north of Fairbanks and 87 km south of Wiseman, recorded the lowest official temperature ever in Alaska of -62 °C. In Fairbanks, the lowest temperature ever recorded was -52 °C, recorded at the Fairbanks International Airport in 1962. This is approximately three kilometers from the on-campus insect enclosure at the University of Alaska Fairbanks. In general, the extent of low temperatures that overwintering insects could experience in Fairbanks can be seen in terms of mean number of days below particular temperature thresholds as measured between 1949 – 2005. During that period, Fairbanks experienced an average of ten days below -40 °C, 44 days below -28.8 °C, 113 days below -18 °C, and 221 days below 0 °C, although the number of days at low temperature extremes is declining (Shulski and Wendler 2007).

While there are a number of official stations that have been recording temperature throughout the state for nearly 100 years (in some locations), the size of Alaska in general and interior Alaska in particular, with its many low-lying valleys, is too great an area to assume that extreme conditions have been adequately monitored. Therefore, the officially recorded extreme minima is most likely an under-representation of low temperatures that overwintering fauna may experience, and unofficial observations suggest that temperatures in the -60s °C have been reached in the Wiseman area (personal comm. Jack Reakoff, Wiseman Alaska). With long winters, the range of low temperature minima ($\leq$ -40 °C), and the large persistent thermal gradient ($>$ 0.1 °C $\cdot$ cm^{-1}) within the

snow pack (LaChapelle 1992; Sturm and Benson 1997), overwintering physiology in Interior Alaska may be at times more properly described as extreme overwintering physiology.

Per (Pete) Scholander

Pete Scholander's work stands at the forefront of comparative physiology not only temporally speaking but also for its far-ranging implications beyond insect physiology. The most direct connection between my work in this dissertation and his relies on his pioneering work in the 1940s and 1950s on insects in Barrow, Alaska. Scholander was the first scientist to examine overwintering strategies of insects in Alaska. In 1947, Scholander measured temperature and respiratory rate on insects (and lichens) in Barrow, Alaska. Since this was the heyday of comparative physiology, after making measurements on high latitude fauna and flora, he felt that a comparison had to be made on lower latitude individuals, so he immediately flew on a military transport to Cuba, where the "air command gave [him] a jeep and [he] drove at night up to the little town of Guantanamo" (Scholander 1990). Here, he collected tropical insects and lichens and brought them back to Barrow. In January 1948, he measured respiratory rate from 0 to 30 °C and did not detect significant differences among insects (or within lichens) from the two regions (Scholander *et al.* 1953).

Although this project was abandoned, he continued to examine ice-trapped insects at the bottom of shallow tundra ponds. In the ice, midge (chironomid) larvae were embedded. As he thawed larvae under warm water, they changed from a yellow to a bright red, and two questions were raised, one of which has had substantial implications

beyond overwintering physiology. First, were these larvae truly frozen and, therefore, freeze tolerant, or were these larvae freeze avoiding and, therefore, overwintering in a supercooled state? This is one of the first instances when the two major overwintering strategies–freeze tolerance and freeze avoidance–were brought into sharp focus, and these strategies continue to be examined today by physiologists. In the last chapter of this dissertation, these two strategies are brought into sharp focus with the discovery of an insect that is capable of simultaneous freeze tolerance and avoidance within an individual. In fact, Scholander's 1948 inquiry was most likely the first time that the overwintering status of insects from Alaska was distinguished. The second question that the embedded larvae raised was gas permeability in ice. In experiments with 0.1 mm layers of ice and known concentration of gasses, Scholander and his group conducted the first micro-gas analysis and showed gas permeability of ice to be low. A later study (EA Hemmingsen 1959) showed oxygen permeability to be even lower than that found by Scholander *et al.* (1953). While Scholander's finding has been very important to the study of fauna and flora overwintering, the greatest influenceof this finding may be on the method of measuring "ancient atmospheres" (Scholander 1990) such as greenhouse gas concentration from air bubbles trapped in ice cores. Micro-gas analysis is routinely used today to estimate carbon dioxide levels in glaciers dating back hundreds of thousands of years in order to more clearly understand past climates and atmospheric CO_2 levels related to global climate issues.

A second interesting historical relationship between Scholander's work and mine is his questioning how polar fishes in ice-leaden seas do not freeze despite the fact that

their plasma's equilibrium freezing point is higher than the sea's freezing point. This question was pursued and answered (antifreeze glycoproteins, AFGP) by Dr. Art DeVries, who was my co-adviser's (Dr. John "Jack" Duman's) major professor. DeVries and Duman's work on antifreeze proteins in fish led to Duman's discovery of insect antifreeze proteins (1977) that was a major factor in the collaboration between Duman and my other co-adviser Dr. Brian M. Barnes (University of Alaska Fairbanks/Institute of Arctic Biology).

Keith Miller

Keith Miller, a retired professor at University of Alaska Fairbanks (UAF) and the Institute of Arctic Biology (IAB), also contributed substantially to understanding of the overwintering physiology of insects. In a conversation with him I explained that one of my goals was to work on an insect that he had not already examined–I'm still searching. Although an exhaustive examination of Miller's published work is beyond the scope of this brief history, three works in particular reveal the breadth of his work and its influence on not only my dissertation but also on the work of Dr. B.M. Barnes (UAF/IAB) and Dr. J.G. Duman (University of Notre Dame). In 1982, Miller published "Cold-hardiness strategies of some adult and immature insects overwintering in interior Alaska." This paper reviewed a range of cold-hardiness factors including freeze tolerance and avoidance, supercooling, lower lethal temperatures, survival, and seasonal changes in polyhydric alcohols. Among these overwintering features, he was able to associate no less than six orders, 15 families, and 17 species of insects from Interior Alaska. This work in particular has been used by me not only as a reference but as a guide.

After finding dual supercooling events in a fungus gnat (see Chapter 4), I consulted this work in order to find which insects from Alaska had already been known to have dual supercooling events. While Miller showed dual supercooling points (SCPs) in some insects, he did not find it in the fungus gnats. I suspect that Miller would have discovered dual freezing events in this gnat had he been less conscientious in examining freezing and survival: after he had found the first freezing event, he re-warmed the bath to see if the gnats survived, which they did. When I called him to discuss my findings, it became clear to me that I had just been lucky enough to be freezing the gnats with other insects that required very low temperatures.

Another direct influence of Miller's work on the Barnes and Duman collaboration is the procedure of reducing bath temperature by 0.2 °C / min. Miller, when examining the adult *Upis ceramboides* found that this freeze-tolerant beetle would survive freezing nearly 100 % when the bath was reduced < 0.3 °C / min down to -62 °C. If the bath temperature had been reduced by 0.35 °C / min, substantial mortality resulted (Miller 1978). Miller and Werner (1987) examined extreme supercooling in three species of freeze–avoiding willow gall insects. These species still hold the supercooling record in Alaska, with some individuals supercooling down to -64 °C. What was also interesting was their association between low supercooling, high molality (4 to 6) glycerol, and low hemolymph melting points (-14 to -18 °C). This paper pointed the way for the work on the extreme supercooling and vitrification in *Cucujus clavipes puniceus* (Chapters 2 and 3).

Mark Oswood

Mark Oswood, a retired professor at UAF/IAB, gave me the idea of working on Odonata (dragonflies) for my M.S. which I pursued with Dr. Pat Doak. My initial interest in overwintering was piqued while taking a course in aquatic entomology with Dr. Oswood, especially when the class was in the field collecting insects and while listening to him and his guest speaker Dr. John "Jock" G. Irons III describe their experiences and experiments with overwintering of freshwater benthic invertebrates (Oswood *et al.* 1991; Irons *et al.* 1992). Like Scholander in the 1940s in Barrow, Dr. Mark Oswood noted that arthropods could be found in frozen substrate (Oswood *et al.* 1991). In personal communications with Oswood, Miller, and Irons, they have also described to me the complexity of the freezing environment in lentic and lotic habitats. Oswood *et al.* (1991) concluded his publication with "Questions and Opportunities" that highlighted the intricacies of the freshwater situation: Year-to-year differences in benthic temperatures, some of which may or may not stress invertebrates; differences in supercooling capacity when animals are frozen in contact with moist vegetation, with water, or under dry condition; the presence of antifreeze proteins. These ideas were helpful when on 20 September 2004, while returning from Toolik Field Station (68° 38'N), we (Duman, Barnes, Walters, and I) found many stoneflies in temporary runoffs during our descent from Atigun Pass. In contrast to many subarctic streams that flow throughout winter (Irons *et al.* 1992), we collected and examined individuals from a stream (the West Fork of the North Fork of the Chandalar River) approximately three km south of Atigun Pass that completely freezes (and even temporarily dries up in summer).

The works by Oswood, Miller, and Irons helped prepare me for my contribution to the Walters *et al.* (2009) paper on the freeze-tolerant stoneflies. One last interesting note on the stoneflies: We began with unidentified individuals. Although they were found in one location, our initial attempts to identify this species were difficult. I contacted a number of individuals who were not interested. After discussing this problem with Dr. Mark Wipfli (UAF/IAB) and Dr. Nick Hughes (UAF/School of Fisheries and Ocean Sciences), I found Dr. Richard Baumann at Brigham Young University who was willing to identify individuals to species. The difficulty in identification of insects, especially in Alaska, is a common theme throughout this work.

Brian Barnes and Jack Duman

As part of a course entitled Physiological Ecology of Overwintering (Biology 623, 1994-1995), Brian Barnes conducted a study (Barnes *et al.* 1996) on the northern green stink bug *Elasmostethus interstinctus* (Heteroptera: Acanthosomatidae, Linnaeus, 1758). In this course, he and his students found that the stink bug successfully overwinters through a combination of supercooling and selection of appropriate overwintering microhabitat; however, this freeze-avoiding insect was susceptible to inoculative freezing by external ice crystals. In fact, they found that inoculative freezing resulted in higher lethal subzero freezing events that were near or higher than microhabitat temperatures, implying that stink bugs must either position themselves so as to avoid contact with ice crystals or suffer substantial mortality. They did not note any unique overwintering posture for these animals, as they had for the freeze-avoiding wasp (*Vespula vulgaris*) that overwinters in the same leaf litter microhabitat as the stink bug.

Overwintering *V. vulgaris* can be found hanging below leaves by grasping vegetation with its mandibles and folding its wings to cover the ventral part of its body to prevent inoculative freezing (Barnes *et al.* 1996). In discussions about this paper, Drs. J.G. Duman and B.M. Barnes began a collaboration in which I have been involved, and it is with a response to this paper that my dissertation begins (Chapter 1).

Barnes and Duman first met at the AAAS (regional) meeting at Denali Park, Alaska, 1999. This meeting resulted in the first of two collaborative NSF grants and my becoming a PhD student under their supervision. My first work included collecting insects and testing for antifreeze proteins (AFPs) as part of a general survey (see Duman *et al.* 2004) of insects from areas north of the Brooks Range to Anchorage. In addition, I had the great pleasure of spending two years at Notre Dame in the Duman lab. I was also responsible for work on the green stinkbug *Elastmostethus interstinctus* (Chapter 1), and it was at Notre Dame that I learned about insect antifreeze isolation. It was near the end of the first grant and my stay at Notre Dame that Duman and I discussed the possibility of examining deep supercooling and vitrification in *Cucujus clavipes puniceus* (Chapters 2 and 3), especially since returning to UAF would allow me to examine these overwintering features at the organismal level. Finally, in 2008, a Fairbanks collecting trip was arranged for a UAF photographer that resulted in a serendipitous examination of the fungus gnat *Exechia nugatoria*, an insect not previously scrutinized by any of us in this collaboration. Examination of the dual freezing events found in this species is a testament to my advisors' willingness to allow me to explore new species, resulting in the finding of a new overwintering strategy "on my own." Obviously, much more could be

stated about the work of Drs. Duman and Barnes, but that will be left to the results of the four principal chapters of the dissertation.

Summary of significant results by insect

Chapter 1 *Elasmostethus interstinctus*: Resistance to Inoculative Freezing

The Barnes *et al.* (1996) paper on two freeze-avoiding insects including the green stink bug *Elasmostethus interstinctus* served as a scientific backdrop for this chapter. I measured seasonal changes in supercooling capacity, dehydration, and thermal hysteresis in relation to microhabitat temperatures and tested susceptibility to inoculative freezing by wrapping moistened paper around individuals and freezing them in direct contact with ice. While Barnes *et al.* (1996) found this species to be susceptible to inoculative freezing as high as -6 °C in the spring, I found an increase in the capacity to resist inoculative freezing to -16 °C. In addition, by measuring the seasonal increase in thermal hysteresis, indicative of the presence of antifreeze proteins, I found that stink bugs were capable of 13 °C of supercooling while in contact with ice below their hemolymph freezing point. Although physiological adjustments help ensure that their supercooling capacity is not exceeded, this freeze-avoiding insect must select appropriate below-snow microhabitats to ensure their supercooling capacity is not surpassed by low temperature minima.

Chapter 2 *Cucujus clavipes puniceus*: Deep Supercooling and Vitrification

Vitrification in the beetle larvae *Cucujus clavipes* was predicted to occur based on earlier work by Bennett *et al.* (2005). My work confirms this hypothesis. First, the

species was found to be a subspecies of *Cucujus clavipes* known as *Cucujus clavipes puniceus,* a western subspecies located in Pacific coastal states and Alaska. It is also a freeze-avoiding insect. The geographical range of this species is not known. In attempting to categorize supercooling as deep supercooling, Bennett *et al.* (2005) and this work found that if larvae avoided freezing to -58 °C, then there was no evidence of freezing as low as the laboratory bath could go (ca. -75 °C). We subsequently used temperature below -58 °C as the threshold to describe deep supercooling. At the same time as testing deep supercooling, we sent larvae that did not freeze at this threshold to 21st Century Medicine (California) to confirm vitrification. Vitrification is the condition whereby the diffusion of water is inhibited and the phase change from a liquid to a solid crystalline state is by-passed. Antifreeze proteins function to inhibit ice nucleation, and through the combination of low water content and high colligative antifreeze (glycerol) concentration, the molecular mobility of remaining water molecules in high viscosity and at low temperatures is arrested. Under the direction of Drs. Greg Fahy and Brian Wowk at 21st Century Medicine (California), we found that larvae did not freeze when the temperature was lowered to -150 °C. We also found that larvae would not freeze upon re-warming, a condition known as devitrification, that can take place when water molecules regain the ability to diffuse. Devitrification is a major stumbling block in vitirifcation processes in general, and my findings may serve an example for future biomimetic research in cryopreservation; furthermore, we found that the highest temperature at which larvae can vitrify is -58 °C, the temperature of our proposed deep supercooling threshold. I do not show that vitrification occurs in nature. However, since

the lowest officially recorded temperature in Alaska is -62 °C (officially recorded at Prospect Creek, south of the northern range of *C.c. puniceus*), I conclude that larvae could potentially vitrify and subsequently overwinter in this condition. This adds vitrification as a third overwintering strategy to the long-standing categories of freeze tolerance and avoidance.

Chapter 3 *Cucujus clavipes puniceus*: Probability of Freezing

In attempting to analyze some of the variables that allow individual *Cucujus clavipes puniceus* to deep supercool, I enlisted the help of Dr. Julie McIntyre (UAF, Department of Mathematics and Statistics). Over many discussions, logistic regression was found to be a method by which I could create a statistical model for determining which parameters are significant in the insect's not freezing. Logistic regression can be used to determine the probability of a given dichotomous variable occurring, (in this case "freeze or not freeze"). In addition, logistic regression has also allowed me to estimate water content leading to the probability that 50 % of larvae would deep supercool rather than freeze. The amount of water at which 50 % of larvae would deep supercool was greater in Wiseman larvae than in the Fairbanks larvae.

Chapter 4 *Exechia nugatoria*: Simultaneous Freeze Tolerance and Avoidance

This chapter on the fungus gnat provides evidence of another overwintering strategy that had not been known: the simultaneous freeze tolerance and avoidance in individual fungus gnats, *Exechia nugatoria*. This fly both tolerates and avoids freezing in different body compartments at temperatures of -30 to -50°C while overwintering in

Fairbanks, Alaska. I suggest that this differential freezing is accomplished by regional dehydration that prevents inoculative freezing between the frozen abdomen and the supercooled thoracic and head compartments. These results should be interesting to a broad range of investigators including biologists, material scientists, and biophysicists, given that this insect illustrates the ability to supercool beyond the homogenous ice nucleation temperature (~ -40°C) and inhibits the propagation of ice at low temperature.

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Chapter 1 Overwintering and inoculative freeze resistance in the freeze-avoiding stink bug *Elasmostethus interstinctus* (Heteroptera: Acanthosomatidae) in Alaska[1]

Abstract

Selection of below snow microhabitat for overwintering by freeze-avoiding insects can reduce exposure to cold by 30 to 40 °C compared to uncovered locations. However, subnivean microhabitats can subject animals to direct contact with ice crystals that may compromise supercooling capacity and result in mortality due to inoculative ice nucleation and freezing. We examined the overwintering behavior and physiology of the freeze-avoiding stink bug *Elasmostethus interstinctus* from Interior Alaska. We found significant seasonal changes in supercooling points and water content of adult insects from summer maxima near -11 °C and 1.8 mg · mg dry mass^{-1} to winter minima near -22.5 °C and 1.3 mg · mg dry mass^{-1}, respectively. We tested susceptibility to inoculative freezing by cooling individuals while in direct contact with ice. Under these conditions, stinkbugs remained unfrozen to a minimum of -18 °C, a level of supercooling that is lower than the microhabitat temperatures they normally encounter in winter. Nevertheless, mortality of insects held in semi-natural conditions increased over the winter suggesting that individuals may experience inoculative freezing.

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[1] Todd Sformo, Kent Walters, Brian M. Barnes, and John G. Duman. 2009. Overwintering and inoculative freeze resistance in the freeze-avoiding stink bug *Elasmostethus interstinctus* (Heteroptera: Acanthosomatidae) in Alaska. Prepared for the journal Physiological and Biochemical Zoology

Introduction

To survive winter, arctic and subarctic arthropods must adapt to low temperatures physiologically and behaviorally. The two predominate physiological strategies are freeze tolerance (the ability to survive freezing of extracellular body water) and freeze avoidance (Bale 1987; Duman 2001; Holmstrup *et al.* 2002). Recently identified in the Alaskan fungus gnat *Exechia migratoria*, a third strategy (more typical of plants) combines both tolerance to ice formation within certain tissues and avoidance of ice in other contiguous, supercooled tissues (Sformo *et al.* 2009).

For freeze-tolerant organisms, the steady-state of being frozen leads to a reduction in metabolic rate that allows significant energy savings over the winter (Lundheim and Zachariassen 1993; Irwin and Lee 2002). Also, frozen individuals are in vapor pressure equilibrium with their surroundings and do not lose water to the environment since there is no gradient driving water from a high to a low vapor pressure (Lundheim and Zachariassen 1993; Irwin and Lee 2002; Zachariassen *et al.* 2004). Ice formation in freeze-tolerant organisms tends to occur at high subzero temperatures between -5 to -12 °C with the aid of ice nucleating factors (Zachariassen 1985; Duman 2001).

Freeze-avoiding organisms avoid spontaneous freezing by removing ice-nucleating factors (Zachariassen 1985; Neven *et al.* 1986), producing antifreeze proteins (AFPs) (Duman 2001) and/or colligative antifreezes such as glycerol (Storey and Storey 1991; Duman 2001; Duman and Serianni 2002), and/or undergoing cryoprotective dehydration (Bayley and Holmstrup 1999; Bennett *et al.* 2005).

Selection of overwintering microhabitat can be an important component in overwintering success by ensuring that temperatures do not exceed the lower limits of freeze tolerance or avoidance (Werner 1978; Marchand 1982; Bale 1987; Hayhoe and Mukerji 1987; Olsen *et al.* 1998). Many freeze-avoiding insects select overwintering sites below snow cover to take advantage of insulating effects of snow, leaf litter, and soil (Danks 1978, 1981; Pruitt 1979; Marchand 1982; Kalliomaki *et al.* 1984; Miller and Werner 1987; Hayhoe and Mukerji 1987; Sturm *et al.* 1995; Benson 2001; Olfert and Weiss 2006). To avoid freezing by innoculative ice nucleation, freeze-avoiding insects also select overwintering sites that minimize contact with ice crystals (Barnes *et al.* 1996).

The northern green stink bug *Elasmostethus interstinctus* Linnaeus, also known as a birch bug, is a hemipteran (Heteroptera: Acanthosomatidae) whose palearctic distribution includes Siberia, northern China and Japan (Barber 1932), northern Europe, the Northwest Territories in Canada, and Alaska (Thomas 1991). This species is also commonly found in southern Finland and Sweden, where it is referred to as a shield bug (Mappes *et al.* 1996). The most northern observation was near Aklavik in the Northwest Territories of Canada close to the "delta of the Mackenzie River" (Barber 1932). In interior Alaska, Barnes *et al.* (1996) showed that this species overwinters as a freeze-avoiding adult, and Duman *et al.* (2004) demonstrated the presence of hemolymph antifreeze protein indicated by thermal hysteresis activity. In this study, we found specimens as far north as treeline on the south side of the Brooks Range (~ 67° 30' N), Alaska. The locations of specimens near the Brooks Range and interior Alaska, two of

the coldest environments in North America (Shulskiand Wendler 2007), suggest that these insects have adapted to thermal challenges throughout their life cycle.

Barnes *et al.* (1996) concluded that adult stink bugs survive Alaska winters through a combination of supercooling and selection of appropriate overwintering microhabitat, since they are susceptible to inoculative freezing by contact with external ice. Inoculative freezing resulted in lethal freezing at temperatures that were near or higher than microhabitat temperatures, implying that stink bugs must either position themselves to avoid contact with ice crystals or suffer substantial mortality. Stink bugs did not adopt a protective overwintering posture, as was observed in the freeze-avoiding yellow jacket wasp (*Vespula vulgaris*) that overwinters in the same leaf litter microhabitat. Overwintering *V. vulgaris* can be found hanging by its mandibles below leaves with wings folded over legs and ventrum (Barnes *et al.* 1996). This earlier study examined autumn and spring individuals and did not include winter adapted insects and consequently did not determine effects of inoculative freezing on the most cold acclimated individuals. The goal of this study was to investigate resistance to inoculative freezing by assessing seasonal changes in wet and dry supercooling capacity, water content, and thermal hysteresis in adult stink bugs from three geographical locations in Alaska: Wiseman, Fairbanks, and Anchorage, a latitudinal gradient of nearly 700 km.

Materials and Methods

Animals

At all study sites, stink bugs descend from birch trees where they have been feeding in the canopy and make their way into leaf litter where they spend the winter. While we

have found only a few stink bugs in the leaf litter in August, they are found in greater numbers in September and October during leaf-fall. Insect are found as individuals or together in small groups under the current year's leaf litter or inside curled-up leaves. Individuals were covered by at least one leaf. We rarely observed stink bugs within the previous year's leaves or near the soil surface. Adults were collected at the end of September through early October in leaf litter in the forest behind the Institute of Arctic Biology on the University of Alaska Fairbanks campus (64° 72'N). This site contains a variety of vegetation, including *Betula papyrifera* (paper birch), *Alnus crispa* (alder), *Salix* spp. (willow), and *Picea glauca* (white spruce). Insects were collected by hand and, for later retrieval during winter, placed inside plastic containers that were placed within the leaf litter on the ground surface in a fenced enclosure 50-300 meters from collecting locations. Other containers were placed 1-2 m above the ground where they remained uncovered by snow. Periodically throughout winter, insects were retrieved from the containers, brought into the laboratory, and tested for supercooling points within one hour. Stink bugs were also collected from near Wiseman (67° 30' N) and at Alaska Pacific University, Anchorage (61° 11'N), Alaska in habitats similar to collecting locations in Fairbanks.

Microhabitat Temperature

We recorded above- and below-snow micro-habitat temperatures with thermister probes attached to Hobo Temperature Dataloggers (Onset Instruments, Pocasset, Mass.) every three hours in 2002-2003 and every 30 minutes in 2004-2006. A probe was placed 1 m

above ground to record ambient conditions and another within the current year's leaf litter to record below-snow temperature at the location of overwintering stink bugs. Since few individuals were collected from Wiseman, temperature data are not provided; however, both examples of above- and below- snow temperatures patterns for this area can be found in Bennett *et al.* (2005).

Supercooling and Survival

To assess supercooling capacity, individuals were placed in either a 0.6 or 1.5 mL micro-centrifuge tube, and a thermocouple was placed against the body, with a small piece of packing foam securing the insect and thermocouple. Up to 16 tubes with individuals were then placed into a beaker that was immersed in an ethanol bath and equilibrated to 0 °C. Thermocouple leads were attached to a computer controlled multi-channel thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, Ohio, USA) that recorded temperature every five seconds. Cooling proceeded at 0.2 °C min^{-1}. The lowest body temperature recorded at the release of the latent heat of fusion, as evidenced by an exotherm, was recorded as the supercooling point (Lee 1991). To determine susceptibility to inoculative freezing during supercooling, supercooling point (SCP) measurements were conducted on individuals while in contact with ice (designated "wet"). After attachment to a thermocouple as above, individuals were surrounded with moist paper towel and placed into the tube. Tubes were equilibrated at -2 to -5 °C until an exotherm indicated that ice had formed within the paper towel. The temperature was

then lowered at 0.2 °C min^{-1} until exotherms associated with the insects freezing were recorded.

We assessed survival of insects under field conditions and after supercooling tests by retrieving insects from containers left above or below snow cover. Insects were placed on moist paper towels held at 2.5 to 4 °C and observed for coordinated movements each day for one week.

Water Content

Body water content was determined according to Rojas *et al.* (1986). Individual larval fresh mass was determined to the nearest 0.1 mg. Larvae were then dried at 60 °C (~ 72 h) to constant dry mass. Water content was calculated as mg · mg^{-1} dry mass (Hadley 1994).

Thermal Hysteresis Activity

Thermal hysteresis (TH) activity, an indication of the presence of antifreeze proteins (AFPs), was determined according to the method of DeVries (1986). Hemolymph samples (~ 1-3 µL) were drawn from individual larva by pricking the pronotum with a 26 ga. needle; hemolymph was collected using a 10 µL capillary tube. The tubes were flame-sealed at one end and sealed with mineral oil at the other, leaving a gap between the hemolymph and oil. The sample was sprayed with an aerosol that partially froze the hemolymph. The sample was placed into a bath where the temperature was raised and lowered to find the melting and freezing points, while observing the crystal using a

stereomicroscope. The melting point was defined as the temperature at which the ice disappeared. Beginning again with a small crystal (~ 0.25 mm), we determined the freezing point by lowering the temperature until the crystal grew. In the absence of AFPs, if the temperature is lowered 0.01 - 0.02 °C below the melting point, the crystal will grow (i.e., the equilibrium melting and freezing points are nearly identical); however, if AFPs are present, the crystal will not grow until the temperature has been lowered to the hysteretic freezing point, at which point crystal growth is rapid (i.e., melting point ≠ freezing point). The difference between melting point and freezing point is the thermal hysteresis activity and is an index of the amount of AFP activity.

Polyol Determinations

Winter acclimated individuals from Anchorage and Fairbanks were bled and hemolymph pooled (N = 52) for determination of polyols. Hemolymph glycerol concentrations were measured by a spectrophotometric (UV) assay (Boehringer Mannheim/R-Biopharm, Marshall MI, USA). The test was calibrated with glycerol standards.

^{13}C NMR was used to determine the presence of polyols and other potentially important solutes in the hemolymph of cold acclimated stink bugs. The ^{13}C $\{^{1}H\}$ NMR spectrum was obtained on a Varian Unity *Plus* 600-MHz NMR spectrometer equipped with dual $^{1}H/^{13}C$ 3-mm microprobe (Nalorac), operating at 150.86 MHz for ^{13}C. The hemolymph sample (250 µL) was diluted with 30 µL of $^{2}H_2O$) and transferred to the NMR tube prior to data collection. Data acquisition conditions were as follows: 31,000 transients; 2.5 s recycle time; 303 K; 1–230 ppm spectral window. The resulting FID

was zero-filled (yielding a final digital resolution of 0.14 Hz/pt), and a 1-Hz line broadening function was applied prior to Fourier transformation. Chemical shifts were externally referenced to the most intense C1/C3 signal (64.2 ppm) observed in the spectrum of a glycerol standard (Kukal *et al.* 1988).

Statistical Analysis

Comparisons of normally distributed data (Shapiro-Wilk test $P > 0.05$, $\alpha = 0.05$) were compared by T-test. The Brown -Forsythe homogeneity of variance test was performed. If variances were found to be heterogeneous ($P < 0.5$), a weighted mean was used for 1-Way ANOVA, with the post hoc Tukey-Kramer Adjustment for Multiple Comparison tests, and is designated "weighted mean" (SAS 9.1, SAS Institute, Inc). The association between water content and SCPs was examined by Spearman Rank Correlation across years by season: Summer months were defined as 21 June –20 September, autumn as 21 September –20 December, and winter as 21 December –20 March, and spring as 21 March –20 June. Unless otherwise noted, all values are given as mean ± standard error of mean, and number (N) of individuals tested (mean ± s.e.m., N).

Results

Animals, Microhabitat, and Survival

In Fairbanks, adult stink bugs emerged from the leaf litter in mid-May, although they were usually not conspicuous until late May–early June when they were frequently seen on birch catkins. Mating was observed mid-June through July. From July through late September, adults and nymphs were difficult to find, and we assume most were in the

upper canopies of trees. By early October, adults were found in the current year's leaf litter, where they overwintered. We do not know whether stink bugs overwinter as adults for more than one winter.

Over the four winters of this study, below-snow temperatures in the leaf litter in Fairbanks were rarely observed to be lower than -15 °C while above-snow air temperatures were regularly < -30 °C (Fig. 1.1). Only once between 2002 – 2006 was below-snow temperature < -15 °C: on 28 January 2006 minimum temperature was -23.5 °C. In Anchorage, between October 2003 – January 2004, the above-snow temperature minimum was -24.3 °C while the below-snow minimum was -14.9 °C. After January, the logger stopped collecting data for an unknown reason. From February 2005 to November 2005, the above-snow minimum at the Anchorage site was -15.0 °C while the below-snow was minimum -11.7 °C.

Under laboratory conditions, stink bugs that froze during SCP measurements showed 100% mortality. Individuals left in containers located above-snow in Fairbanks also showed 100 % mortality, while survival rates of stink bugs located in containers placed below-snow varied. On 10 January 2003, 18 of 21 individuals collected in Fairbanks and held below-snow were alive. We held 15 of 18 of the live individuals for one week in the lab, and within that time 12 of 15 took flight. The lowest below-snow temperature by this date had been -14.1 °C, while above-snow temperature was as low as -37 °C. However, only 18 of 40 insects retrieved on 1 April 2003 from containers held below-snow were alive, even though the lowest below-snow temperature was -14.4 °C, recorded on 10 January 2003. In comparison, 100% (16 of 16) survival was found in

2004 – 2005 season of Fairbanks-collected adults held in Fairbanks below-snow individuals and retrieved on 15 April 2005. Minimum below-snow temperature that year was -10.6 °C in late December (Fig. 1.1).

Stink bugs collected in Fairbanks were also held over the winter in containers placed above- and below-snow in Wiseman, Alaska, near the northern limit of their distribution. On 5 April 2003, all above-snow individuals were dead, but 73 of 100 below-snow individuals were alive. Minimum below-snow temperature was -13.0 °C in late October. For the remainder of winter, below-snow temperatures were -2 to -8 °C, and it was not until late March that below-snow temperatures decreased below -10 °C again.

Supercooling and Water Content

Among the three populations, values for dry supercooling points (insects cooled without contact with ice) and water contents (WC) were not significantly different in January 2003. Wet (insects cooled in contact with ice) and dry supercooling points were not significantly different in June 2003 or in September 2003 in Fairbanks. Water contents were not significantly different in September 2003 between Fairbanks and Anchorage, except for a slight (0.1 mg · mg^{-1} dry) but significantly different WC in June 2003. Since supercooling points and water contents did not significantly vary during overwintering months in the Fairbanks and Anchorage populations, we combined data from all individuals in these locations for the rest of the study.

Mean dry supercooling points determined in stink bugs collected from Fairbanks and Anchorage (2002-2005) declined by 10 to 12 °C in winter and spring, respectively, from a summer high SCP of -12.1 °C (Table 1.1). The lowest individual SCP in this study was -27.4 °C. Body water content also decreased from a summer high of 1.8 mg · mg dry mass^{-1} to 1.3 mg · mg dry mass^{-1} in autumn and spring. The lowest average WC occurred in winter (1.1 mg · mg dry mass^{-1}) and represents a loss of 0.6 mg · mg dry mass^{-1} (Table 1.1).

Dry supercooling points decreased between September 2004 and February 2005 by 5.7 °C (combined averages) (Table 1.2). Wet supercooling points over the same period varied by 8.6 °C (combined averages). The subzero wet supercooling points between September and November 2004 were significantly higher than the dry supercooling points at the same time. It was not until 24 January 2005 that the wet supercooling points declined and were found not to be significantly different than dry supercooling points between September – January. Although there was a trend toward a lower wet mean supercooling point in February, there was also a further decrease in the mean dry supercooling temperature (Table 1.2).

The most extensive collection of stink bugs in one location occurred in Fairbanks from autumn 2004 through the spring of 2006. Mean supercooling points and water contents are shown in relationship to above- and below-snow temperatures (Fig. 1.1). Mean supercooling points reached the lowest values (i.e., the highest supercooling capacity) of -22.9 and -23.5 °C on 12 March 2005 and 23 March 2006, respectively. Supercooling points rose in May 2005 and remained high through August 2005, with

means varying by testing date between -8.2 to -15.3 °C. Low water contents were recorded by October 2004, and they did not statistically differ through April 2005 and between September 2005 through March 2006, with a range in means by testing date between 1.1 to 1.4 mg · mg dry mass^{-1}. Mean water contents, which varied between 1.7 to 2.4 mg · mg dry mass^{-1}, rose in May and remained high through August 2005.

Polyol Determination

While glycerol tends to be the most commonly accumulated polyol in the hemolymph of many overwintering insects, the colorimetric assay did not detect glycerol in the winter hemolymph of *E. interstinctus*. ^{13}C NMR analysis of winter hemolymph confirmed the absence of glycerol, and instead indicated that a α,α-trehalose accumulated in the winter hemolymph (results not shown). The presence of six approximately equally intense ^{13}C-signals in the winter hemolymph, which exhibited chemical shifts characteristic of C1-C6 of α,α-trehalose (Kukal *et al.*, 1988), unambiguously supported the presence of trehalose. Peak heights of the trehalose signals were approximately ten times greater than those of any other low molecular mass organic solutes.

Correlation between water content and SCP

There was a significant positive association between WC and SCP during summer months ($\rho = 0.1944$, $P < 0.01$, N = 155) with WC explaining approximately 6 % of the variation in SCP; however, no significant associations were found in autumn ($\rho = 0.0300$, $P = 0.69$, N = 177) or winter ($\rho = -0.007$, $P = 0.9461$, N = 88).

Thermal Hysteresis and Melting Points

Mean hemolymph TH values increased by 2 to 3 °C between summer and winter (Table 1.3), an indication of synthesis and secretion of antifreeze proteins (AFPs). These values combine measurements within months and across years (2002-2004). Hemolymph melting point temperatures decreased significantly from summer to winter, signifying an accumulation of low molecular mass solutes. The decrease in hemolymph freezing points from summer to winter reflected the combined effects of lower melting points and increased thermal hysteresis. Thermal hysteresis values plateaued October-January reaching mean values of 2.4 °C. Although it appears that MP, FP, and TH continued to decline after January, the loss of body water made it difficult to collect hemolymph. Even cutting legs and antennae and centrifuging insects to force hemolymph out did not produce useful samples. This difficulty in obtaining hemolymph in February and March resulted in too few samples to include in the statistical analysis.

Discussion

In this study of overwintering strategies in adult green stink bugs, we found that supercooling point values of insects tested dry, while not in contact with ice, decreased from summer values of -12.1 °C to winter values of -22.7 °C. This increase in supercooling capacity is correlated with accumulation of trehalose and antifreeze protein within the hemolymph. The leaf-litter microhabitat where stinkbugs overwinter is usually insulated by snow against low air temperatures that were found regularly to exceed dry winter supercooling capacity. Despite increased supercooling capacity in

winter, survival rates of stink bugs placed below the snow decreased from 45 % (April 2003), while minimum below-snow temperatures varied by year: -14.0 °C (2002 – 2003), -15.0 °C (2003 – 2004), -11.5 °C (2004 – 2005), and -23.5 °C (2005 – 2006). When tested while in contact with ice, insects froze at values ranging from high subzero temperatures near -8.2 °C in autumn to -18.3 °C in winter, suggesting that inoculative freezing continues to be a threat to freeze-avoiding stink bugs over the long Interior Alaska winter.

Mean dry supercooling points demonstrated in this study (-18 °C in autumn and -23 °C in spring) are lower than values shown previously for *E. interstinctus* stink bugs from the same area. Barnes *et al.* (1996) reported dry supercooling points in autumn-collected adults of -9 °C and -17 °C in spring. Differences in pre-treatment and handling of individuals for testing between the two studies may account for these differences. Autumn-collected individuals in Barnes *et al.* (1996) were acclimated in the laboratory at 4 °C in constant darkness for two to three weeks before testing. In this current study, insects were acclimatized under more naturally changing conditions of temperature, humidity, and photoperiod in an outside enclosure, which may have led to enhanced levels of cold-hardiness. Our testing for supercooling points by placing insects in contact with thermocouple junctions involved less manipulation of individuals as compared to Barnes *et al.* (1996). They used beeswax to secure thermocouple junctions to insects, potentially increasing susceptibility to freezing due to abrasion of cuticle or breaking of fine cuticular hairs (Bennett *et al.* 2005).

We anticipated that decreases in water content would correlate with increases in supercooling capacity of stink bugs in winter, but we did not find this to be the case. The lack of a relation between WC and SCPs is not unusual, however. While the western subspecies of flat red bark beetle larvae, *Cucujus clavipes puniceus*, also found in Interior Alaska, loses substantial body water and increases supercooling capacity during winter, the eastern subspecies *Cucujus clavipes clavipes* from Indiana (~ 41° 45' N) does not dehydrate in winter but supercools to approximately -23.0 °C by increasing colligative antifreezes and noncolligative AFPs (Bennett *et al.* 2005). Water loss in stinkbugs in late August – September (Fig. 1.1) may be coincident with cessation of feeding on birch catkins. In additional, there was a small but significant decline in WC and SCPs from autumn to winter (Table 1.2). The mechanism of water loss at this time of the year is most likely evaporation due to differential vapor pressure between the insect and the frozen environment (Lundheim and Zachariassen 1993; Zachariassen *et al.* 2004). At subzero temperatures, supercooled body fluids have higher vapor pressure than surrounding air. Consequently, insects lose water to the environment until vapor pressure between the supercooled fluid and the frozen microhabitat come to equilibrium (Lundheim and Zachariassen 1993; Bayley and Holmstrup 1999).

Water loss increases solute concentration and lowers the equilibrium melting-freezing point (Zachariassen, 1985; Holmstrup *et al.* 2002). Melting points decreased from -0.4 °C in summer to -1.8 °C to -1.9 °C in autumn and winter, respectively. Calculated from the melting points in summer vs. autumn or winter measurements, stink bugs hemolymph osmolality increased by 0.7 - 0.8 Osmol., representing approximately a

25 - 28 % increase. That trehalose signals were approximately ten-fold more intense than other low molecular weight solutes observed in the hemolymph suggests that accumulation of trehalose as a predominant osmolyte explains most of the melting point depression. Although glycerol tends to be the most commonly accumulated solute in winter by insects (Sömme and Block 1991), trehalose, the common blood sugar in insects (Storey and Storey 1991), has also been found to accumulate in response to changes in water content and temperature. Under extreme desiccation, trehalose stabilizes membranes by replacing water via binding to the headgroups in phospholipid bilayers (Crowe *et al.* 1997) in a variety of arthropods such as Collembola (Worland *et al.* 1998), tardigrades (Westh and Ramløv 1991; Hengherr *et al.* 2007), and chironomid larvae *Polypedilum vanderplanki* (Watanabe *et al.* 2002). In terms of overwintering, a temperature-dependent accumulation of trehalose has been shown in the silkworm *Philosamia cynthia* (Hayakawa and Chino 1981 and 1982) and in the soybean pod borer *Leguminvora glycinivorella* (Shimada *et al.* 1984) has been shown to stabilize proteins. As a protein-stabilizing osmolyte, trehalose functions entropically: it is accommodated within bulk water and is predominately excluded from the protein surface. This preferential exclusion of trehalose away from the protein surface forces the hydration of the protein that maintains native protein conformation (Hochachka and Somero 2002).

Accumulation of colligative antifreeze (trehalose) and noncolligative AFPs in autumn is correlated to lower dry supercooling values (-22 °C) in all three of the populations examined; however, stink bugs must still find appropriate snow covered microhabitats to successfully overwinter, since all insects placed above snow died. Even

when insects were placed below snow, survivorship varied between 86 to 55 % for minimum winter microhabitat temperatures down to approximately -14 °C. These results suggest that some stink bugs are still susceptible to inoculative freezing. Although we did not directly determine the minimum temperature that stink bugs survive under snow cover, we found the -14 °C below-snow temperature is comparable to below-snow temperatures found in Fairbanks in previous years (Barnes *et al.* 1996; Bennett *et al.* 2005), but it is a lower temperature than is noted for taiga forests in Interior Alaska, where Sturm *et al.* (1995) reported temperature ranges at snow-ground interface of -3 to -5 °C. If stink bugs were more typically confronted with these higher temperatures, then even the wet supercooling value of -9.7 °C found in September (Table 3) would most likely afford more survival.

The presence of antifreeze proteins, indicated by hemolymph thermal hysteresis, did not significantly vary from October through January (Table 4). Olsen *et al.* (1998) found AFPs to be effective against inoculative freezing. In their study, the beetle larvae *Dendroides canadensis* (Olsen *et al.* 1998) increased in resistance to inoculative freezing during winter. In addition to hemolymph antifreeze proteins, immunofluorescence that identified antifreeze proteins showed the presence of AFPs in the epidermis underlying the cuticle in winter that was not present in summer. The beetle showed 11.9 °C of protection against external ice inoculation below the hysteretic freezing point in hemolymph. In our study, we showed approximately the same level of protection for stink bugs in winter, a wet SCP 13.2°C below the hysteretic freezing point (Table 1.3).

In autumn, the surfaces of leaves in the litter have various degrees of wetting, but even during rainy conditions, stink bugs remain dry under the leaf litter. When frost forms or when snow falls, the outer surfaces of leaves that accumulate the various forms of solid state water, but stink bugs are protected from direct contact by the leaf litter. During the autumn/winter period, the substrate is frozen, and individual stink bugs can be in contact with ice; but by then (October), stink bugs have already reached maximum thermal hysteresis (Table 1.4), have decreased water content to winter levels, and displayed resistance to inoculative freezing to temperatures of -8 to -10 °C (Table 1.3). As winter proceeds, a thermal gradient between the air/snow and the soil/snow interface leads to recrystallization (Sturm *et al.* 1995; Benson 2001) and the rearrangement of ice crystals into depth hoar that decreases the number of crystals leading to fewer but larger crystals. The decrease in number and the surface area to volume ratio of ice crystals should reduce the area of contact between a crystal and the insect, leading to lower susceptibility to inoculative freezing (Leather *et al.* 1993; Olsen *et al.* 1998). Under the snow where stink bugs overwinter, the metamorphoses of ice crystals into depth hoar may lead to a drying out of microhabitat and be a reason for the discrepancy between the current study and the earlier work by Barnes *et al.* (1996). While the contact between cuticle and ice was not determined in either study, it could be that our wrapping was not as form fitting around an individual as we had thought, leading to less contact. Conversely, the use of surface snow by Barnes and colleagues, while finer than our wrapping method, is most likely not characteristic of ice crystals at the base of the snow pack. In fact, great care must be taken so that snow does not melt from room temperature

exposure or from a slightly warmed insect itself, leading to water on the surface that may be able to more efficiently inoculate an insect as the water re-freezes.

There appear to be three components to overwintering success in stink bugs: below-snow conditions, contact moisture, and the presence of antifreezes. Stink bugs must be below the snow to insulate themselves from low air temperatures that regularly exceed supercooling capacity. Once under snow cover, stink bugs have the greatest ability to decrease SCPs with colligative and noncolligative antifreezes under dry conditions, and only once during the years of this study did below-snow temperatures exceed dry supercooling ability; however, dry below-snow habitat may not always be found, especially with the constant movement of water vapor under the snowpack. Contact moisture associated with ice crystals at the ground level compromises supercooling capacity, potentially leading to higher "wet" supercooling values. Though there is a progression toward both lower dry and wet SCPs as winter proceeds, susceptibility to inoculative freezing remains relatively high. Excluding the extreme -23.5 °C below-snow temperature, we show that at best stink bugs have a margin of supercooling ability of about 3 to 7 °C under wet conditions. At worst, low below-snow temperatures can be lower than the insects' ability to supercool under wet conditions. Of course, one assumption is that stink bugs remain in constant contact with ice crystals throughout the winter, but the microclimatology of ice crystals in interior Alaska may reduce the probability of contact due to the temperature gradient within the snowpack. The metamorphoses of snow into hoar frost at the base of the snowpack as a result of recrystallization results in the multitude of small (1–5 mm) stellar ice crystals, with high

surface area to volume ratio, being reduced in favor of fewer but larger (6–25 mm) prism-like crystals with a smaller surface area to volume ratio. This transformation takes place throughout winter, reaching the largest size in midwinter (Sturm and Benson 1997). The change in number and shape of crystals over the course of the winter may reduce the incidence of inoculative freezing in the stink bug. The next task will be to not only carefully excavate overwintering stink bugs through winter to determine the exoskeleton-ice surface interface but also to devise repeatable procedures to simulate ice crystal formation on cuticle at various times of the winter in order to estimate more precisely the inoculative freezing effect.

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Figure

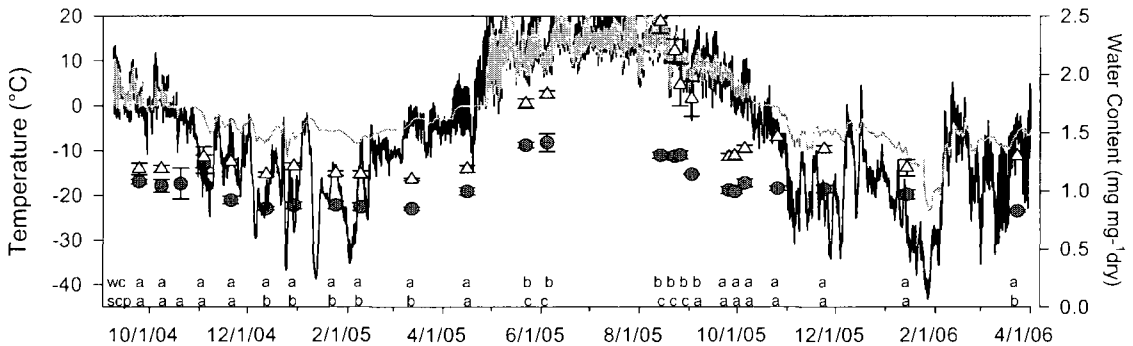

Figure 1.1 Microhabitat temperatures at the overwintering enclosure on the University of Alaska Fairbanks campus in Fairbanks, Alaska (2004–2006). Below-snow temperature is represented by the grey line, and above-snow temperature is represented by the black line. Mean (± sem) water content (cyan triangles) and supercooling points (blue circles) of Fairbanks adults are shown in relation to temperatures. Different letters indicate significant difference in means ($P < 0.05$) with the Tukey-Kramer adjustment for multiple comparisons within WC or SCP. All insects, except for those measured in August 2005, are individuals collected and held at the collecting site. August 2005 measurements were field-collected and immediately tested.
Abbreviations: wc = water content; scp = supercooling.

Tables

Table 1.1 Dry supercooling points (SCP, °C) and water content (WC, mg · mg^{-1} dry) for pooled data from Fairbanks and Anchorage (2002–2005). For each variable, the Brown-Forsythe test indicated heterogeneity of variance ($P < 0.001$); therefore, statistical significance is based on weighted means. Superscript letters and numbers indicate significant difference in means ($P < 0.05$) using Tukey-Kramer adjustment for multiple comparisons, with in SCP or WC.

	SCP Mean ± s.e.m. (N)	WC Mean ± s.e.m. (N)
Summer	-12.1[a] ± 0.03 (201)	1.8[1] ± 0.03 (256)
Autumn	-17.9[b] ± 0.3 (216)	1.3[2] ± 0.02 (216)
Winter	-22.0[c] ± 0.3 (151)	1.1[3] ± 0.02 (127)

Table 1.2 Weighted mean of supercooling points (SCP, °C) from both dry and wet (ice present) supercooling trials from autumn to winter 2004 – 2005. Water content did not significantly differ ($P > 0.1$) between wet and dry insects among these dates, so values were combined ($1.2 \pm 0.01 mg \cdot mg^{-1}$ dry, $N = 154$). Also presented are mean above- and below- snow temperatures along with minimum and maximum temperatures (°C) by month at the collecting location during these trials. Superscript letters indicate significant difference in means ($P < 0.05$) using Tukey-Kramer adjustment for multiple comparisons. Note that "N" under below-snow min, max is the same for above-snow.

Date	Dry SCP ± s.e.m., N	Wet SCP ± s.e.m., N	Above-Snow temperature	Above-Snow min, max	Below-Snow temperature	Below-Snow min, max
09/24/04	-17.0^{a} ± 0.78, 32	-9.7^{c} ± 0.9, 38	1.6 ± 0.3	-10.8, 13.4	2.3 ± 0.2	-1.4, 10.4
10/14/04	-17.6^{ab} ± 1.8, 14	-8.2^{c} ± 0.8, 16	-1.8 ± 0.3	-15.9, 11.9	0.2 ± 0.1	-2.6, 5.2
11/20/04	-18.0^{ab} ± 1.2, 26	-10.3^{c} ± 1.8,10	-12.5 ± 0.3	-24.8, -2.6	-4.0 ± 0.1	-6.4, -1.0
01/24/05	-21.6^{b} ± 0.8, 19	-15.7^{abcd} ± 2.7, 8	-18.6 ± 0.7	-38.9, -1.7	-5.7 ± 0.1	-6.9, -3.0
02/08/05	-22.7^{b} ± 0.9, 16	-18.3^{abd} ± 1.0, 8	-16.1 ± 0.7	-35.4, -4.4	-6.7 ± 0.1	-8.6, -5.2

Table 1.3 Mean melting (MP) and freezing (FP) points and thermal hysteresis (TH) (all °C) pooled by month from 2002–2004 from Fairbanks specimens. Superscript letters indicate significant difference in means ($P < 0.01$) within the MP, FP, and TH categories using Tukey-Kramer adjustment for multiple comparisons.

Month	MP ± s.e.m., n	FP ± s.e.m., n	TH ± s.e.m., n
June	$-0.4^{a} \pm .01$, 16	$-0.6^{a} \pm .04$, 16	$0.2^{a} \pm .03$, 16
September	$-1.0^{b} \pm 0.1$, 23	$-1.8^{b} \pm 0.2$, 21	$0.8^{b} \pm 0.1$, 21
October	$-1.1^{b} \pm .04$, 13	$-3.5^{c} \pm 0.3$, 13	$2.4^{c} \pm 0.3$, 13
November	$-1.8^{bc} \pm 0.3$, 12	$-4.1^{c} \pm 0.4$, 10	$2.4^{c} \pm 0.3$, 10
January	$-1.9^{bc} \pm 0.2$, 15	$-4.4^{c} \pm 0.4$, 15	$2.4^{c} \pm 0.3$, 15
February	-2.9 ± 0.9, 2	-5.9 ± 0.1, 2	3.1 ± 0.9, 2
March	-2.0 ± 0.1, 2	-4.7 ± 0.1, 2	2.7 ± 0.0, 2

Chapter 2 Deep Supercooling, Vitrification, and Limited Survival to -100°C in the Alaskan Beetle Larvae *Cucujus clavipes puniceus* (Coleoptera: Cucujidae)[1]

Summary

Larvae of the freeze-avoiding beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) in Alaska have mean supercooling points in winter of -35 to -42°C, with the lowest supercooling point recorded for an individual of -58°C. We previously noted that some larvae did not freeze when cooled to -80°C, and we speculated that these larvae vitrified. Here we present evidence through differential scanning calorimetry that *C. c. puniceus* larvae transition into a glass-like state at temperatures < -58°C and can avoid freezing to at least -150°C. This novel finding adds vitrification as an insect overwintering strategy. While overwintering beneath the bark of fallen trees, *C. c. puniceus* larvae may experience low ambient temperatures in the -40s°C (and lower) when microhabitat is un-insulated due to low snow cover. Decreasing temperatures in winter are correlated with loss of body water from summer high levels near 2.0 to winter lows levels near 0.4 mg · mg^{-1} dry mass with concomitant increase in hemolymph measures of glycerol concentrations (4 - 6 M) and thermal hysteresis. Finally, we provide direct evidence that Cucujus from Wiseman, Alaska, survive temperatures to -100°C.

Key Words: SCP, supercooling point; AFP, antifreeze protein; DSC, differential scanning calorimetry; TH, thermal hysteresis

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[1] Todd Sformo, Kent Walters, Kennan Jeannet, Brian Wowk, Greg Fahy, Brian M. Barnes, and John G. Duman. *Journal of Experimental Biology* (In Press).

Introduction

The red flat bark beetle, *Cucujus clavipes*, has a broad latitudinal range in North America that extends from above the Arctic Circle (~ 68°N) to North Carolina (~ 35°N), and exists as a western subspecies (*C. c. puniceus* Mannerheim) that occurs in Alaska through to the Pacific coast and as an eastern subspecies (*C. c. clavipes* Fabricius) that extends east from the Great Plains (Thomas 2002). Consequently, investigations of this species present an opportunity to study insect overwintering physiology over a large latitudinal expanse including Interior Alaska, one of the coldest environments in North America.

Insects that overwinter in freezing regions survive either by being freeze tolerant (able to survive the freezing of their extra-cellular water) or freeze avoiding (Zachariassen 1985; Bale 1987; Storey and Storey 1988; Block 1990; Danks, 1991; Duman *et al.*, 1991a; Lee and Denlinger, 1991), and, in one case, simultaneous freeze tolerance and avoidance (Sformo *et al.*, 2009). Insects, and certain other terrestrial arthropods such as collembola, avoid freezing by use of combinations of molar concentrations of cryoprotectant polyols such as glycerol, antifreeze proteins (Duman, 2001), removal and/or masking of ice nucleators (Neven *et al.*, 1986; Duman, 2001), and/or dehydration (Rickards *et al.*, 1987; Lundheim and Zachariassen, 1993; Worland, 1996; Holmstrup and Sömme, 1998; Worland *et al.*, 1998; Danks, 2000; Block, 2003; Worland and Block, 2003). Some collembola and earthworm cocoons (Holmstrup *et al.*, 2002) and the Antarctic midge *Belgica antarctica* (Elnitsky *et al.*, 2008) cryoprotectively

undergo extreme water loss by dehydrating until they are in vapor pressure equilibrium with surrounding ice and therefore do not freeze.

Overwintering adaptations are exaggerated in insects from arctic and subarctic regions, where temperatures can reach below -60°C (Danks, 1981; Miller, 1982; Ring, 1982; Sömme and Block, 1991). Alaska populations of *C. c. puniceus* larvae are known to be freeze avoiding and routinely supercool to group means near -40°C, with individuals supercooling to as low as -58°C (Bennett *et al.*, 2005); in some cases larvae did not freeze when cooled to -80°C in that study. This level of supercooling appeared irregularly among larvae collected near Fairbanks, AK (64° 72'N) but appeared more often in larvae collected further north near Wiseman, AK (67° 37'N) in late November to March. Factors contributing to the ability of *C. c. puniceus* to supercool to low temperatures identified in that study are production of antifreeze proteins (AFPs), accumulation of glycerol, diapause (reduced metabolism in winter), and extensive dehydration. In contrast to the Alaska *C. c. puniceus, C. c. clavipes* larvae from northern Indiana (41° 45'N) show mean winter supercooling points (SCPs) of -23°C and do not dehydrate or diapause in winter (Bennett *et al.*, 2005).

Dehydration contributes to the ability to supercool to low temperatures in Alaska *C. c. puniceus,* in part, by causing the concentrations of AFPs and glycerol to increase (by as much as 5-fold) over the levels synthesized prior to dehydration and by decreasing the amount of water in the insect that is available for freezing (Bennett *et al.*, 2005). Antifreeze proteins can mask ice nucleators and also inhibit inoculative freezing initiated by external ice in contact with the cuticle. AFPs enhance supercooling by both these

mechanisms in larvae of the beetle *Dendroides canadensis* (Olsen *et al.*, 1998; Olsen and Duman, 1997a,b; Duman, 2002). *C. c. puniceus* produce a family of AFPs that are similar but not identical to those of *D. canadensis* (Duman *et al.*, 1998, 2004; Andorfer and Duman, 2000). The extreme desiccation of *C. c. puniceus* makes it impossible to sample hemolymph from animals in mid-winter. However, when hemolymph was collected in the autumn (after AFPs and glycerol had been produced, but prior to dehydration) and then concentrated 3.2-fold to reflect a level of dehydration near that of winter larvae, the concentrated hemolymph exhibited nearly 13°C of thermal hysteresis (Bennett *et al.*, 2005). This is the highest thermal hysteresis ever measured in association with any organism. Thermal hysteresis activity (THA) is the difference between the freezing and melting points of the sample and reflects the amount of depression of the freezing point caused by the AFP, in the presence of ice, below the melting point (DeVries, 1986). However, the level of protection afforded to whole insects by AFPs generally greatly exceeds the magnitude of THA that can be measured in the hemolymph (Zachariassen and Husby, 1982; Olsen *et al.*, 1998; Duman, 2001, 2002; Duman *et al.*, 2004). Consequently, the protection provided by AFPs in *C. c. puniceus* larvae is probably much greater than even the level of THA demonstrated in the concentrated hemolymph.

While a few northern or alpine insects have been shown to have supercool points of -40 to -60°C, not much is known about the mechanisms that contribute to this ability. Three freeze-avoiding Alaska and Canadian Rocky Mountain species that overwinter in willow galls in exposed branches had mean SCPs of -56 to -58°C, with individual SCPs

to -63°C (Miller and Werner, 1987; Miller, 1982; Ring and Tesar, 1980). Mean SCPs of -54°C have been reported in larvae of the beetle *Pytho deplanatus* that overwinter under the bark of fallen spruce trees in the Canadian Rockies (Ring 1982). These levels of freeze-avoidance have been attributed to high concentrations of polyols and removal of ice nucleators and are described as extreme or deep supercooling; however, an ability to deep supercool to below -65°C has not previously been demonstrated in insects. This paper examines the deep supercooling capacity, defined as cooling without freezing below -58°C, of *C. c. puniceus* larvae in Alaska. We examine correlations among body water, glycerol concentration, thermal hysteresis, and ambient temperature to identify physiological and abiotic factors associated with deep supercooling. We demonstrate that *C. c. puniceus* larvae can avoid freezing to -150°C, their body water vitrifies (turns to glass) at a mean temperature of approximately -76°C, and larvae can survive exposure to -100°C.

Methods

Insect Collection and Microhabitat Characteristics

We collected *C. c. puniceus* larvae from under the bark of standing dead *Poplar* spp. trees near Wiseman and Fairbanks, AK, in Septembers of 2005–2007. Larvae were placed in plastic food storage containers (20 x 15 x 10 cm; 20 –150 per box) perforated for gas exchange, along with moist bark from their host trees. Insects were left to acclimatize to local winter conditions by placing the containers either on the ground or suspended (so as not to be covered by snow) for 1–4 months either in undisturbed wooded areas on the University of Alaska Fairbanks campus or near Wiseman. Air and

microhabitat temperatures at these overwintering locations were monitored using Hobo Pro Series data loggers and downloaded with BoxCar Pro 4 software (Onset Computer Corporation, Bourne, Massachusetts, USA).

Supercooling Points

Insects were recovered from containers at different times in winter and brought into the laboratory at the Institute of Arctic Biology in Fairbanks to determine the supercooling point (SCP) and water content. Insects retrieved from Wiseman were kept at -18 to -20°C during transport to Fairbanks and tested within nine hours. Larvae were tested for individual SCP by placing a thermocouple junction (copper-constantan, 36 gauge) against their body in a 0.6 ml plastic tube. Thermocouple leads, monitoring up to 16 larvae at a time, were attached to a computer controlled multi-channel thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, Ohio, USA) that recorded temperature every five seconds. Closed tubes were placed inside a 500 ml glass beaker that was covered and mostly submerged in an alcohol-water cooling bath. Once the temperature of the insects equilibrated to 0°C, bath temperature was reduced at 0.2 °C/min, typically to -60 to -70°C. Before and after supercooling runs were performed, each thermocouple-attachment was visually inspected to ensure that the thermocouple junction was in direct contact with the insect. The lowest body temperature recorded at the release of the latent heat of fusion, as evidenced by an exotherm in the temperature recording, was recorded as the SCP; and since *C. c. puniceus* is a freeze-sensitive insect (Bennett *et al.*, 2005), the SCP is also the lower lethal temperature. Larvae that did not exhibit an exotherm when cooled to -60°C or lower were recorded as deep supercooling.

Water Content

Individual larvae were weighed to the nearest 0.1 mg and then dried at 60°C (48 h) to constant mass. Absolute body water content (WC) was calculated as mg water · mg^{-1} dry mass (Rojas *et al.*, 1986; Hadley, 1994).

Glycerol

Individuals were randomly chosen from those sampled during the 2005 and 2006 field seasons and, based on supercooling point determinations, categorized as freezing (exotherm present) or deep supercooling. Fresh and dry mass (nearest 0.1 mg) were recorded, and individuals were homogenized in distilled water at a 1:1000 dilution. Samples were centrifuged and the supernatant removed. Twenty microliter sub-samples were removed and further diluted by a factor of ten and analyzed for glycerol content using the Boehringer-Mannheim Glycerol Kit. This method measures the amount of NADH oxidized to NAD+ that is stoichiometrically proportional to the initial glycerol concentration of the sample. The glycerol concentration of each individual was calculated based on the glycerol determination and the body water content, assuming equal distribution of glycerol. Glycerol standards were run to check the accuracy of the procedure.

Thermal Hysteresis

In winter, hemolymph cannot be sampled due to the dehydrated state of insects. Consequently, homogenates of larvae were prepared to compare thermal hysteresis activities. Dry larvae were homogenized in a volume of water equal to 100 times the body water. This solution was further diluted 1:7 with 40 mM phosphate buffer (pH 7.5),

and subsamples were tested for thermal hysteresis using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) (Chakrabartty and Hew, 1991). A micrometer syringe delivered 25–100 nl of sample into heavy mineral oil located in the sample well of the osmometer. The sample was initially frozen by cooling to -40°C and then warmed until a single small ice crystal remained. Temperatures were increased by 0.01–0.02°C until the crystal disappeared as assessed visually, thus determining the melting point temperature. This routine was repeated at temperatures below the melting point with a single small crystal present, and the temperature was slowly lowered until the crystal grew; this is the freezing point temperature.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a technique used to measure the change in heat capacity that is indicative of transitions to or from a vitrified or glassy state. The phase transition of a liquid to a vitreous state occurs at the glass transition temperature in the absence of a change between a liquid and solid state. To determine the glass transition temperature, an insect is sealed in an aluminum sample pan within a chamber that also contains a reference pan with no insect present. The two pans are heated and cooled to maintain equivalent temperatures. This requires differential heating and cooling due to the presence of the insect. By monitoring heat flow and change in temperature over time, the unit of heat capacity is measured. When a liquid transitions to a vitreous condition, there is solidification with a concomitant change in heat capacity but without release of the latent heat of crystallization (Fahy, 1995).

When supercooling tests demonstrated that larvae retrieved from the field could deep supercool to -70°C, additional individuals from a second collection container were packed on ice and shipped overnight from Fairbanks to 21st Century Medicine in Fontana, CA. There, a Perkin-Elmer DSC 7 with Pyris software tested for vitrification of insects through differential scanning calorimetry. The temperature scale of the instrument was calibrated by measuring the onset of the crystal transition of cyclohexane at -87.06°C and the temperature when ice water melts while warming at 1 °C/minute. Heat flow was calibrated by measuring the area under the melting curve of a known mass of water ice. Four larvae of approximately 10 mg mass each were folded and sealed in individual aluminum sample pans (Perkin-Elmer part no. 0219-0062). Each larva was cooled from +10 to -150 °C at a rate of 1 °C/minute, and then warmed to +10°C at a rate of 40 °C/min. Thermograms obtained at this high warming rate provided high sensitivity to detect small phase transitions. Additionally, each sample was cooled to -150°C at a rate of 100 °C/min, and thermograms were obtained during warming to +10°C at 5°C or 10 °C /min. Three smaller larvae (< 2 mg) that deep supercooled were tested separately by cooling from +10°C to -150°C at a rate of 10 °C /minute and then warming back to +10°C at a 10 °C/minute.

Survival

In winter 2005-2006, if a majority of insects taken from the outdoor enclosure on a specific date did not freeze when cooled to below -60°C, additional insects from the same group were cooled in plastic tubes at 1 °C/minute to -80 or -100°C. Insects in this second trial that did not display an exotherm were warmed at the same rate to -16°C and

placed back into the container onto cold (-18°C) bark either in a plastic bag (N=12) or not (N=33). Containers were then returned to the outdoor enclosure and replaced under snow. On 1 May, containers were warmed to 20°C, and insects were assessed for survival and activity over a one week period. In winter 2007–2008, after larvae from the field containers exhibited deep supercooling, whole containers with larvae on bark and within plastic bags were cooled at 1 °C/minute to -80 or -100°C before being returned to the field, and larvae were checked for survival in April.

Statistical Analysis

Normally distributed data (Shapiro-Wilk test $P > 0.05$) were compared by T-test or Tukey-Kramer for multiple comparison; non-normally distributed data were compared with a Mann-Whitney and Chi-Square tests (SAS 9.1, SAS Institute, Inc). Unless otherwise noted, values are presented as mean ± s.e.m. To assess association, the Spearman Rank Correlation test was used with averaged rank used for tied observations. Since winter values for mean SCPs and body water content of larvae collected in Wiseman and held either in Wiseman or Fairbanks did not differ (Mann-Whitney, $P > 0.2$), these data were combined and are collectively referred to as the Wiseman population.

Results

Microhabitat Characteristics

Below freezing temperatures began in mid-August in Wiseman and in mid-September in Fairbanks and extended to mid-April in Fairbanks and mid-May in Wiseman (see Bennett *et al.*, 2005 for more detail of conditions at these two sites).

Although air temperatures regularly decreased to lower than -40°C, insulating snow cover resulted in minimum ground temperatures typically near -20°C in both locations. In Wiseman during 2006–2007, however, low snow cover resulted in similar conditions below and above the snow for much of the winter (Fig. 2.1). On 7 Jan 2007, for example, above-snow temperature was -43°C and below-snow temperature was -40°C. In summer, there were several transient cold snaps in Wiseman including on 4 June 2006 when ambient temperatures reached near -5°C. Above- and below- snow temperatures are presented for Fairbanks from October to April 2005–2006 and 2007–2008 in Fig. 2.2.

Supercooling Points and Water Content

Changes in SCP, WC, and ambient temperature over three overwintering seasons showed generally the same trends (Fig. 2.2 and positive correlations Table 2.1) (Note that these data do not include larvae that deep supercooled and did not freeze). As ambient temperatures decreased between summer and autumn, mean supercooling points determined in *Cucujus* larvae collected from Fairbanks and Wiseman populations declined by 20°C from values as high as -7.0°C and by a further 6 to 7°C between autumn and winter to average seasonal minima of approximately -37°C (Table 2.1). The lowest individual SCP was -54.3°C. Body water content of larvae also decreased between summer and winter from 2.1 to 0.8 mg · mg dry mass^{-1} (averages combined for both locations, Table 2.1). Associations between SCPs and WCs by season and location are reported as correlation coefficients (Table 2.1), and, except for the summer Wiseman value, all were significant ($P < 0.0001$). Results restricted to dates when both freezing and deep supercooling occurred in the same experimental run indicate that variation in

water content of Fairbanks larvae explained approximately 24% of the variation in SCP ($P < 0.001$, $\rho = 0.4976$, n = 175). For Wiseman larvae, water content explained approximately 41% of the variation in SCP ($P < 0.001$, $\rho = 0.6393$, n = 202). Overall, mean SCPs decreased with mean WC, when values from insects collected at both sites throughout the study were compared (Fig. 2.3).

Deep Supercooling and Vitrification

Over half (111 of 210; 52.8%) of the *Cucujus* larvae from the Wiseman population did not exhibit an exotherm when cooled to -60 to -70°C during November to February 2005–2008 (Table 2.2). Over half (84 of 165; 50.9%) of the larvae from the Fairbanks population also did not exhibit an exotherm when cooled to -60 to -70°C during December to March 2005–2008 (Table 2.2). We refer to this result as deep supercooling, and we believe these individuals enter a stable, non-crystalline vitreous state. When individuals from Fairbanks and Wiseman were tested on the same day during December to February 2007, 18 of 26 (69.2%) Fairbanks individuals and 11 of 34 (32.3%) Wiseman insects deep supercooled (Chi-Square $\chi^2 = 2.74$; df=1, $P = 0.09$). There was no significant difference ($P > 0.05$, Tukey-Kramer adjustment for multiple comparisons test) between WC of individuals that deep supercooled between populations: Fairbanks WC was 0.4 ± 0.02, n=52 (winter), and Wiseman WC was 0.3 ± 0.02, n=32 (autumn) and 0.4 ± 0.02, n=59 (winter).

Compared to larvae that froze, individuals that deep supercooled had half of the water content, a 1.57-fold greater concentration of glycerol, and a 1.16-fold greater level of thermal hysteresis (Table 2.3), all statistically significant differences.

Differential Scanning Calorimetry

No evidence of the formation of ice in deep supercooled *C. c. puniceus* larvae was observed by differential scanning calorimetry when larvae were cooled to -150°C at all cooling and warming rates studied. The most sensitive test used for detection of freezing events was cooling at 1 °C/min, followed by warming at 40 °C/min. The rapid warming rate increases detection sensitivity for melting peaks because a faster warming rate requires larger heat flow, helping raise small heat flow changes above the noise level (Saunders *et al.*, 2004). In the experience of one of the authors (B. Wowk), the detection sensitivity of the instrument analysis software at this warming rate is approximately 0.05 J/g, corresponding to a mass of ice equal to 0.015% the sample mass.

Thermograms (change in heat capacity as a function of temperature) obtained during warming of four large larvae at 40 °C/min show a large change in heat capacity within a narrow temperature range that indicates a transition to glass (Fig. 2.4). Glass transition or vitrification temperatures, indicated as the thermogram inflection point while warming at 5 °C/min, are presented in Table 2.4. Two large larvae showed a small glass transition near -97 ±1°C followed by a larger transition near -70°C. All four of the large larvae showed a consistent large glass transition at -76°C ±1°C. The three small larvae (1.7 to 3.3 mg) showed higher and more variable glass transition temperatures. In addition, the glass transitions appeared at the same temperature (accounting for a few degrees scanning lag) whether scanning up or down (warming or cooling) through the glass transition.

The glass transition temperature of aqueous solutions in general increases with increasing solute concentration, and the higher glass transition temperature of the smaller larvae suggests the possibility of greater drying due to their greater surface-area-to-volume ratio; furthermore, the two separate glass transitions seen in some animals are indicative of two separate fluid compartments, one with a higher water concentration that undergoes a glass transition at a lower temperature, and one with a lower water concentration that transitions at a higher temperature.

Survival

Survivorship (2005–2006) of larvae from Fairbanks and Wiseman left below-snow overwinter was 93% and 92%, respectively, when assessed in the spring of each year. In 2007–2008, Fairbanks and Wiseman individuals maintained above the snow in Fairbanks showed 57 and 80% survival, respectively, while 90% of below-snow individuals survived in both groups.

On 18 January 2006, larvae (N =16) from Wiseman held in Fairbanks were cooled in contact with individual thermocouples to -72°C at 1 °C/min. No exotherms occurred. Twelve of these were placed on bark, sealed in a plastic bag, and returned to the outside enclosure. On 1 May 2006, 6 of 12 were alive. On 20 December 2007 half of Wiseman larvae retrieved from the outdoor enclosure (N = 8) deep supercooled, and half froze when cooled to -71°C. The next day, 41 additional larvae were retrieved from the same enclosure and cooled to -100°C at 1°C/min. These larvae were held below -58°C (the lowest exotherm temperature we have measured) for approximately 84 minutes. After reaching -100°C, they were warmed at 1 °C/min to approximately -25°C and then

returned to the enclosure, as above. On 7 April 2008, 3 of 41 were alive. No other insects (N =138) survived deep supercooling from -70 to -100°C. Note that we have not yet directly shown that specimens found to vitrify based on differential scanning calorimetry are alive after rewarming, but our data appear to be compatible with the possibility of such a demonstration in the future.

Discussion

This study provides the first evidence of an insect avoiding freezing in extreme low temperatures by entering a stable, non-crystalline vitreous condition that is, at least in some individuals, survivable. Differential scanning calorimetry of rapidly cooled individuals reflected vitrification (glass transition) at temperatures as high as -58°C. This vitrified fluid is well-protected against freezing, showing no detectable tendency to form ice. The fact that these larvae can vitrify, however, does not mean that all survive these low temperatures. Half of the larvae cooled between -70 to -73°C survived, while only 7% cooled to -100°C survived. One possible reason for the lower survivorship between -70 to -100°C may have to do with our method of transporting larvae from the enclosure to the low temperature bath and back to the enclosure. Larvae were deposited in an outside enclosure in September and were typically retrieved for testing when ambient conditions were < -20°C (late November through March). Many of the containers that held the insects were frozen to the ground. To retrieve insects, the containers had to be forcefully removed. This jostling may cause already supercooled insects to nucleate or become mechanically damaged and thus be a source of mortality. Similar manipulations and rapid temperature changes in the laboratory also may be damaging.

Fairbanks and Wiseman, locations in interior Alaska, provide excellent low temperature settings to examine extreme overwintering physiology of insects. Both locations have some of the coldest environments in North America with official recordings of -52°C in Fairbanks in 1962 and -62°C in 1971 at Prospect Creek Camp (Shulskiand Wendler, 2007) 87 km south of Wiseman and approximately 107 km south of the northern limit of *C. c. puniceus*. These minima are likely an under representation of temperatures that *C. c. puniceus* as a species have experienced, both historically and in recent years. Unofficial evidence suggests that temperatures in the -60s°C have recently been reached in the Wiseman area. This is low enough to expose *C. c. puniceus* to their highest glass transition temperature as recorded in the present study. Low ambient temperatures are usually buffered by an insulating layer of snow; however, there are years when snow cover is minimal, resulting in below-snow temperatures that are comparable to above-snow temperatures (Fig. 2.1).

Regardless of location, supercooled *C. c. punicius* larvae were regularly found in direct contact with ice crystals for months at a time and at low temperatures (Bennett *et al.*, 2005). Since larvae were found not to be at increased risk of inoculative freezing (Bennett *et al.*, 2005), direct ice contact and low temperature may enhance dehydration, which we assume is taking place through differential vapor pressure between the unfrozen insect and external ice. This process has been noted in other organisms such as earthworm cocoons (*Dendrobaena octaedra*) and collembola (*Onychiurus arcticus*) (Holmstrup *et al.*, 2002), and the Antarctic midge, *Belgica antarctica* (Elnitsky *et al.*, 2008). The direct contact between ice and the body of the larva reduces the diffusion

distance, which is inversely proportional to water loss rate (Holmstrup and Zachariassen, 1996). Exposure to low temperatures increases differential vapor pressure between the frozen microhabitat and the supercooled body fluids; consequently, these organisms lose water to the environment until they come to a new equilibrium. It is problematic whether *C. c. puniceus* larvae reach vapor pressure equilibrium with the environment at the extremely low temperatures to which they are exposed, and consequently, they may not correctly be said to undergo "cryoprotective dehydration" that requires vapor pressure equilibrium (Holmstrup *et al.*, 2002). However, dehydration results in the low water contents that are found in larvae in winter, especially those that deep supercool. For Fairbanks larvae, the range of water contents associated with deep supercooling was 0.6 to 0.2 mg · mg^{-1} dry mass (compared to 2.0 mg · mg^{-1} dry mass in summer) and 0.9 to 0.3 mg · mg^{-1} dry mass for the Wiseman population. There is no significant difference between mean water content (approximately 0.4 mg · mg^{-1} dry mass) between populations.

In addition to decreasing the amount of water available for freezing, dehydration also causes concentration of solutes. There was a 1.6-fold increase in mean glycerol concentration in individuals that did not freeze as compared to insects that froze, with one individual having a 6.5 M glycerol concentration (Table 2.3). Although we did not directly measure antifreeze protein concentration, there was also a 1.2-fold increase in thermal hysteresis activity (a proxy for the presence and concentration of antifreeze proteins) in diluted homogenates of unfrozen individuals compared to those of individuals that froze. In general, the amount of dehydration measured could cause the

concentration of antifreezes to increase by as much as 5-fold; therefore, AFPs not only contribute to supercooling ability of non-deep supercooling individuals, but they also almost certainly contribute to the ability of individuals to deep supercool and vitrify. The inability to sample larval hemolymph, due to their extreme desiccation in winter, precludes the determination of hemolymph thermal hysteresis, therefore necessitating the measurement of thermal hysteresis in homogenates. As a result we do not know the thermal hysteresis activity of winter hemolymph; however, Bennett *et al.* (2005) reported earlier that hemolymph from autumn larvae prior to desiccation exhibited nearly 13°C of thermal hysteresis when concentrated 3.2x to reflect a level of dehydration experienced by winter larvae under a lesser level of dehydration. The known ability of AFPs nearly identical to those of *C.c. puniceus* to inhibit ice nucleators (Duman, 2001, 2002) indicates that the *Cucujus* AFPs assist deep supercooling and vitrification.

Although dehydration in Alaska *C. clavipes* larvae contributes to cold hardiness, many insect species do not readily lose body water over the winter period yet still show a seasonal increase in supercooling capacity (Zachariassen, 1985; Duman *et al.*, 1991a; Bennett *et al.*, 2005). Even the eastern subspecies of *Cucujus* from northern Indiana (~ 41° 45'N) does not dehydrate in winter but supercools to approximately -23.0°C (Bennett *et al.*, 2005). In contrast, the western subspecies investigated in this study loses body water and increases supercooling capacity during winter, with the annual minimum supercooling point for the eastern subspecies being achieved by the western subspecies in Alaska in October, prior to dehydration. For other insects, like the western subspecies of *C. clavipes*, increased supercooling capacity is correlated to a decrease in body water,

although vapor pressure equilibrium, and therefore strict cryoprotective dehydration, may not be attained (Lundheim and Zachariassen, 1993; Gehrken, 1989; Rickards *et al.*, 1987; Leather *et al.*, 1993; Block, 2003; Worland and Block, 2003; Danks, 2000; Bennett *et al.*, 2005). In fact, the association between water content and supercooling in this study indicates that Fairbanks populations maintain a moderate association (R^2 = 24%) between SCP and WC in autumn and winter. For the Wiseman population, the association increases from 24% in the autumn to near 40% in winter. Zachariassen *et al.* (2004a,b) explain the correlation between log body mass and supercooling point in freeze-avoiding insects by citing the work of Bigg (1953), who found a linear relationship between supercooling and the logarithm of water volume: as log mass (and volume of water) declines, supercooling capacity increases. As a consequence of low water content through dehydration and high solute concentration, the diffusion of water molecules should be inhibited at some low temperature, allowing the remaining water to turn to glass, a situation that has been described as a "viscous slowing down of supercooling liquid" (Tarjus and Kivelson, 2000). We believe that this low temperature threshold in *C.c. puniceus* larvae is approximately -58°C. Bennett *et al.* (2005) and this study did not record any freezing events in *Cucujus* larvae at temperatures < -58°C.

The present study provides direct evidence through differential scanning calorimetry that a glass transition temperature can occur at -58°C. Through dehydration tolerance, the combination of noncolligative AFPs and colligative antifreeze (glycerol), and by the depression of the homogeneous nucleation temperature of body water to below the glass transition temperature (Fahy *et al.*, 1984), *C. c. punicius* larvae should

not be threatened with ice nucleation, even under extreme low ambient temperatures and in direct contact with ice crystals. Theoretically, vitrified larvae should be stable for long periods of time due to the unique properties of a vitrified substance. Larvae in a vitrified condition will not have the stress of volume expansion and grain growth of ice in tissues (Hochachka and Somero, 2002), and since the vitrified condition encompasses both intra- and extra-cellular fluids, vitrified larvae should not encounter the differential osmotic and ionic stress (Storey, 2004) that is associated with concentration of solutes when extra-cellular water freezes. It is interesting that Holmstrup *et al.*, (2002) also demonstrated that ice formation did not occur in dehydrated collembola and earthworm cocoons cooled to -60°C, although vitrification was not reported.

We suggest that over a broad range of temperatures, *C. c. puniceus* larvae overwinter through a continuum of supercooling capacities by which they survive high latitude winters in a freeze-avoiding state. Production of AFPs in early autumn, followed by cessation of feeding and clearing of the gut, lower the SCPs into the -20°C range. Then between autumn and winter glycerol accumulates, body water decreases, and there is a concomitant increase in solutes (including glycerol and antifreeze proteins) that affords individuals low temperature freezing resistance to approximately -40°C. As body water declines to near 0.4 mg · mg^{-1} dry mass, the additional increase in solutes (which in one case reached approximately 6 M glycerol) has the additional benefit of increasing viscosity, leading to a threshold for vitrification of approximately -58°C. We have measured hundreds of nucleation temperatures in winter *C.c. puniceus* larvae, and -58°C is the lowest SCP that we have recorded, a temperature which is consistent with the

highest glass transition temperature measured in larvae. For temperatures lower than -58°C, the combination of low body water, increase in viscosity, and low temperatures promote vitrification of body fluid that augments survival of individuals well beyond temperatures officially recorded in Alaska, putatively adding vitrification to the list of potential insect overwintering strategies. While most *C. c. puniceus* larvae overwintering *in situ* are covered by an insulating snow cover for much of the winter, this is not always the case (Fig. 2.1). Although our evidence of low temperature survival indicates that individuals can survive temperatures lower than the lowest glass transition range, we did not directly test survival and vitrification simultaneously. The next tasks are to examine whether larvae truly vitrify in nature and whether they are capable of surviving vitrification under laboratory conditions.

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Figures

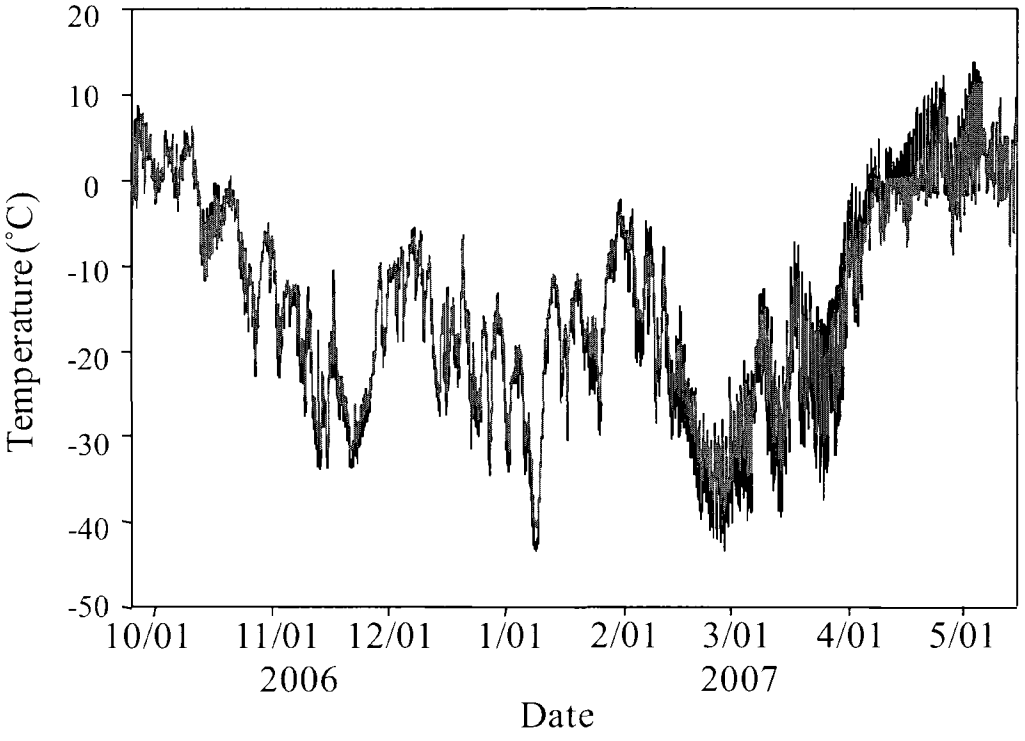

Figure 2.1 Above- and below- snow temperatures (black and red lines, respectively) from Wiseman 2006-2007. This figure indicates similarity between above- and below-snow temperatures when snow accumulation is low.

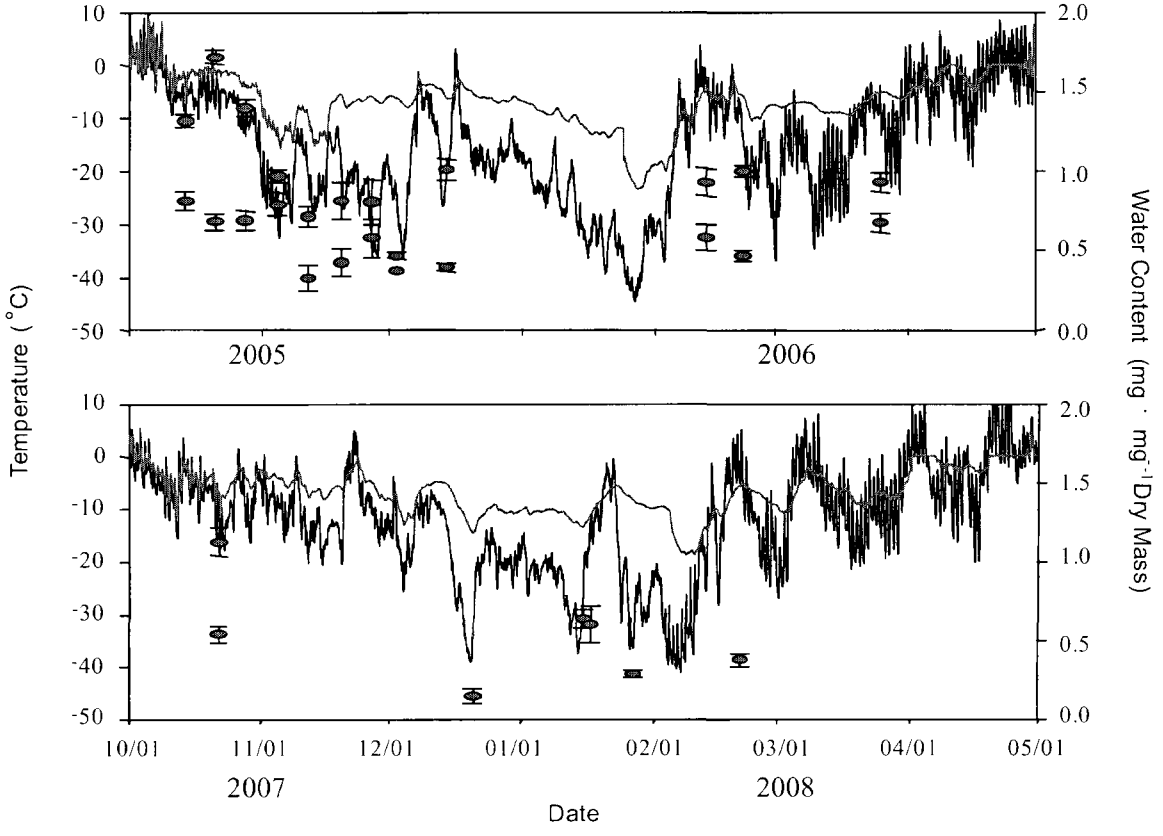

Figure 2.2 Above- and below- snow temperatures (black and red lines, respectively) from Fairbanks, mean (± s.e.m.) supercooling points (blue ovals), and water content (red ovals) of Wiseman C. c. puniceus larvae held in Fairbanks during October to May 2005–06 and 2007–08. Note that on some of these dates larvae deep supercooled (did not freeze), and these are not included in the data shown.

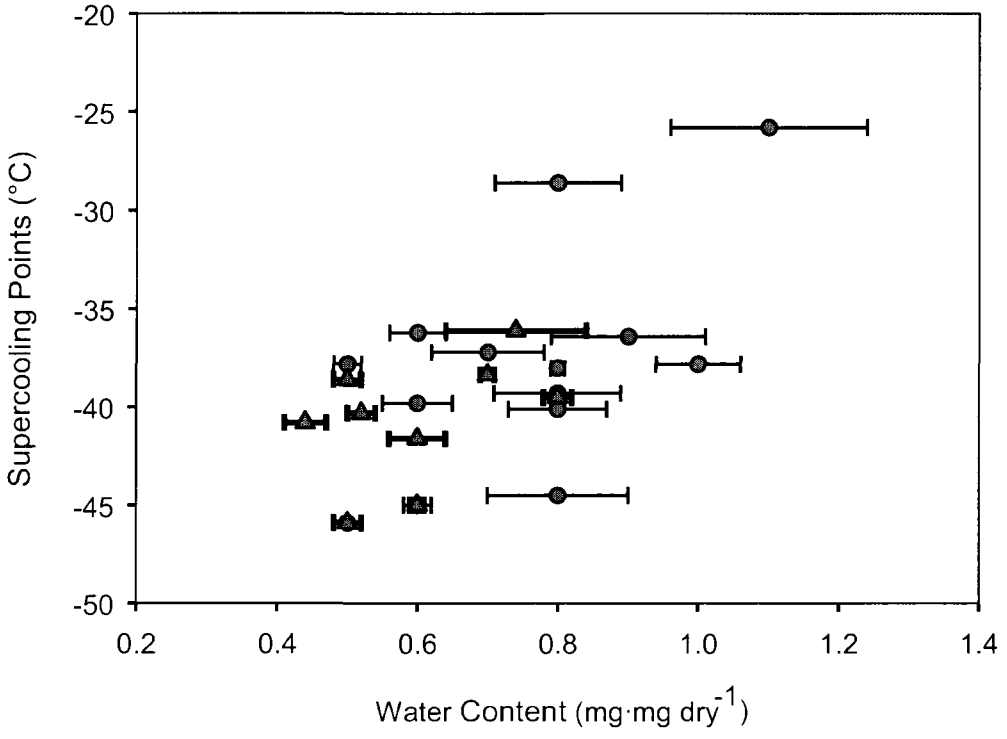

Figure 2.3 Mean supercooling points and water content (± s.e.m.) of larvae from Fairbanks (blue triangles) and Wiseman (red circles). Data are from November to February trials, when individuals were also capable of deep supercooling (no exotherm < -60 to -70°C); however, these deep supercooling larvae are not included in these data.

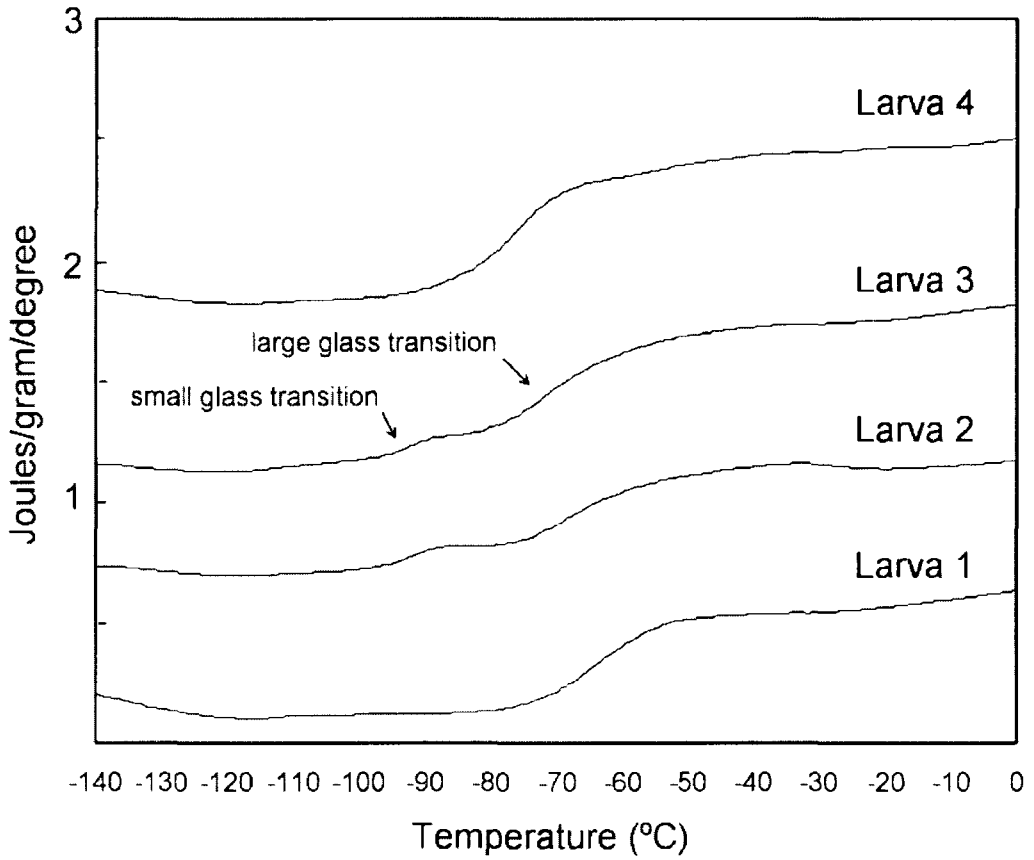

Figure 2.4 DSC thermogram shows warming thermograms of larvae. The warming rate was 40 °C/minute. The thermogram heat flows are normalized to specific heat capacity units. The starting point on the vertical scale for each thermogram is arbitrary. Two of the four larvae exhibited a double glass transition.

Tables

Table 2.1 Seasonal changes in mean (±s.e.m. (N)) supercooling points (°C) and water content (mg · mg^{-1} dry mass) of insects that froze (exotherm). Note that these data do not include deep supercooling individuals.

Season	Months	Loc	Supercooling point	Water Content (exotherm)	Correlation Coefficient
Summer	May - September	F	$-8.2^{a} \pm 0.3$ (170)	$2.0^{d} \pm 0.04$ (169)	0.36
	July	W	$-7.0^{a} \pm 0.3$ (21)	$2.2^{d} \pm 0.2$ (21)	NS
Autumn	October-December	F	$-28.1^{b} \pm 1.0$ (65)	$1.4^{e} \pm 0.05$ (65)	0.48
	October-November	W	$-30.0^{b} \pm 1.0$ (91)	$1.2^{f} \pm 0.04$ (91)	0.35
Winter	December - April	F	$-36.4^{c} \pm 0.7$ (174)	$0.8^{g} \pm 0.03$ (175)	0.49
	December - March	W	$-38.7^{c} \pm 0.8$ (168)	$0.8^{g} \pm 0.02$ (170)	0.62

Abbreviations: Loc = population; F = Fairbanks; W = Wiseman Superscript letters indicate significant difference in column means ($P < 0.05$) with Tukey-Kramer adjustment for multiple comparisons.

Table 2.2 Proportion of individuals that deep supercooled compared to total tested per supercooling run by month and year of larvae from both locations and mean water content (WC, mg · mg^{-1} dry mass) of individuals that deep supercooled.

Fairbanks			Wiseman		
Date	Deep supercooling/ total	WC (deep supercooling)	Date	Deep supercooling/ total	WC (deep supercooling)
December 05	1/12	0.6	November 05	5/13	0.6
January 06	10/10	0.2	November 05	3/15	0.4
March 06	1/14	0.6	November 05	9/16	0.4
December 06	5/11	0.4	November 06	19/24	0.3
December 06	7/7	0.5	December 05	12/13	0.4
January 07	1/11	0.4	December 05	2/12	0.9
February 07	1/15	0.4	January 07	4/13	0.4
February 07	4/6	0.4	February 07	2/16	0.5
March 07	14/16	0.4	February 07	10/12	0.4
December 07	10/14	0.2	February 07	7/8	0.3
December 07	1/12	0.2	December 07	11/29	NA
January 08	8/9	0.3	January 08	8/8	0.4
March 08	21/28	NA	January 08	12/16	NA
			January 08	7/15	NA
Grand Mean ± s.e.m. WC		0.38 ± 0.04			0.45 ± 0.05

Table 2.3 Mean (±s.e.m. (N)) body water, glycerol, and thermal hysteresis levels in larvae that froze or did not freeze. Individuals were sampled October to December 2005 and February to March 2007. Superscript numbers indicate statistical test used, while superscript letters indicate significant difference ($P < 0.05$).

	Supercooling point (°C)	Water Content[1] (mg · mg^{-1} dry)	Glycerol[2] (M)	Thermal Hysteresis[3] (°C)
Exotherm	-39.5 ± 1.6 (39)	0.8 ± 0.07^{a} (39)	2.8 ± 0.19^{a} (37)	2.07 ± 0.11^{a} (21)
No exotherm	Did not freeze (36)	0.4 ± 0.13^{b} (36)	4.4 ± 0.13^{b} (36)	2.39 ± 0.08^{b} (21)

[1]Mann-Whitney, $U = 796.5$, $P < 0.0001$

[2]T-test with unequal variance, $t = 6.73$, df = 63.6, $P < 0.0001$

[3]T-test with equal variance, $t = 2.33$, df = 40, $P = 0.02$

Table 2.4 Temperatures of glass transition of Cucujus clavipes puniceus larvae.

Individuals	Mass (mg)	Large glass transition temp. (°C)	Small glass[1] transition temp. (°C)	Magnitude (J/g/K) of large glass transition	Magnitude (J/g/K) of small glass transition
1	1.7	-66[2]	not obs	0.6	not obs
2	2.3	-58[2]	not obs	0.6	not obs
3	3.3	-71[2]	not obs	0.6	not obs
4	6.5	-76[3]	not obs	0.6	not obs
5	10	-75[3]	-96	0.45	0.15
6	10.6	-76[3]	not obs	0.6	not obs
7	12	-76[2]	-98	0.45	0.15

[1]The small glass transition temperature, which could not be detected while warming at 5 °C/min, was obtained from the 40 °C/min thermogram and adjusted for thermal lag by subtracting the temperature difference between the large glass transition events on the 40 °C/min and 5 °C/min thermograms.

[2]Glass transition temperature was measured while warming at 10 °C/min.

[3]Glass transition temperature was measured while warming at 5 °C/min.

Chapter 3 Probability of Freezing in the Freeze-Avoiding Larvae of the Beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) from Interior Alaska[1]

Abstract

Freeze-avoiding insects must resist freezing or die. A suite of low-temperature adaptations, including the production of noncolligative antifreeze proteins, colligative antifreezes (polyols), and dehydration, allows most individuals to resist freezing below the lowest ambient temperatures experienced *in situ*. Despite these adaptations, some individuals freeze at temperatures slightly below ambient, while others resist freezing significantly (20 to 50 °C and greater) below ambient temperatures. We used logistic regression to estimate the probability of freezing in larvae of the freeze-avoiding beetle *Cucujus clavipes puniceus*. We hypothesized that water content ≤ 0.5 mg · mg^{-1} dry mass would lead to deep supercooling (resistance to freezing below < -58 °C). We found a significant interaction between water content and ambient below-snow temperature and a significant difference between individuals collected from Wiseman and Fairbanks, Alaska. Individuals from Wiseman deep supercooled with greater water content and over a greater range of ambient temperatures than individuals from Fairbanks, leading to significantly different lethal water content associated with 50 % probability of freezing.

Key Words: SCP, supercooling point; logistic regression; exotherm; LD50

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[1]Sformo T, Mueller-Stoffels M, Walters K, Duman JD, Barnes BM, McIntyre J. Submitted to Journal of Insect Physiology

Introduction

All organisms subjected to subzero temperatures face potentially lethal freezing events, whether due to ice formation, ice crystal growth, or cellular dehydration and associated increases in solute concentration (Zachariassen 1985; Costanzo *et al.* 1995; Duman 2001; Wolfe and Bryant 1999); however, the stochastic nature of ice nucleation makes prediction of the temperature of ice formation and the extent of supercooling difficult (Dorsey 1948; Bigg 1953; Knight 1967; Vali 1995). While there is agreement on the homogeneous ice nucleation temperature range of -39 to -41 °C for small volumes of pure water, biogenic ice formation most likely proceeds by heterogeneous ice nucleation (for a contrary view, see Zachariassen *et al.* 2004). In heterogeneous ice nucleation, organic, inorganic, and even surface impurities of containers holding aqueous solutions can function as nucleation sites. Impurities and sites can catalyze ice formation at subzero temperatures substantially higher than -40 °C, and especially active ice nucleators initiate ice only slightly below the equilibrium freezing point. Investigations on overwintering organisms such as terrestrial invertebrates have occasionally reported supercooling ability below the homogenous ice nucleation temperature of water (Miller 1982; Ring 1982; Bennett *et al.* 2005; Sformo *et al.* 2009). For example, recently it was demonstrated (Sformo *et al.* 2009; chapter 2) that larvae of the beetle *Cucujus clavipes puniceus* from Alaska can deep supercool to temperatures well below -40 °C by entering a vitreous state.

Overwintering insects adapt physiologically by becoming freeze tolerant (able to survive the freezing of their extra-cellular water), freeze avoiding (Zachariassen 1985;

Bale 1987; Storey and Storey 1988; Block 1990; Danks, 1991; Duman *et al.* 1991; Lee and Denlinger 1991), and, in one documented case, through a combination of simultaneous freeze tolerance and avoidance in which individuals survive ice formation in the abdomen, while inhibiting freezing in the head and thorax by supercooling (Sformo *et al.* 2009). For freeze-tolerant insects, ice nucleation of the extracellular water is a protective mechanism, inhibiting lethal intracellular ice, and allowing significant energy and water savings over the course of winter (Lundheim and Zachariassen 1993; Zachariassen *et al.* 2004). Ice formation in many freeze-tolerant organisms tends to occur at high subzero temperatures, between -5 °C and -12 °C in insects, and generally with the aid of ice nucleating proteins (Zachariassen 1985; Duman 2001). Ice nucleation at high subzero temperatures leads to slower freezing rates (Mazur 1984) and to the formation of a lower amounts of extracellular ice (Mazur 1984). Freeze-avoiding organisms, on the other hand, must avoid freezing at the lowest ambient temperatures that occur within their microhabitat. To avoid freezing, organisms are known to remove ice-nucleating factors (Zachariassen 1985; Duman 2001), produce antifreeze proteins (AFPs) (Duman 2001), accumulate colligative antifreezes like glycerol (Storey and Storey 1991; Duman 2001; Duman and Serianni 2002), and employ dehydration (Bayley and Holmstrup *et al.* 1999; Bennett *et al.* 2005).

Overwintering insects may enhance their survival beyond what is provided by physiological mechanisms by appropriate selection of microhabitat. Microhabitat selection can substantially ameliorate low temperatures experienced over the winter (Werner 1978; Marchand 1982; Hayhoe and Mukerji 1987). However, some freeze-

tolerant organisms are thought to select above-snow locations to minimize the insulating effects of snow (Miller 1982; Bennett *et al.* 2005) resulting in the earliest possible onset of ice nucleation, leading to longer periods of time frozen in order to save water and energy (Bale 1987). Freeze-avoiding insects, on the other hand, are thought to select below-snow sites to mollify the extremes in low winter temperatures and decrease wide variability in temperatures through the insulating effects of snow and the warmer temperatures associated with ground cover (Danks 1991; Pruitt 1979; Marchand 1982; Kalliomaki *et al.* 1984; Miller and Werner 1987). A few exceptions (Miller and Werner 1987) have been noted, including Bennett *et al.* (2005) who found the freeze-avoiding beetle larvae *Cucujus clavipes* from Wiseman and Fairbanks (64° 72' N), Alaska, overwintering both above- and below-snow; yet, survivorship in either location was high (90 %) even when temperatures were in the -40s °C.

A recent study on the red flat bark beetle *C. c. puniceus* (see Chapter 2) showed that larvae under experimental conditions entered a vitrified (glassy) state at temperatures between -58 to -80 °C and 7 % of larvae survived after exposure to -100 °C at a cooling rate of 1 °C/min. Factors contributing to this ability to deep supercool (temperatures < -58 °C) were production of antifreeze proteins (AFPs), accumulation of glycerol, diapause (reduced winter metabolism, in Bennett *et al.* 2005), and extensive dehydration. The larval stage of this species transitions from autumn to winter conditions by losing body water and increasing solutes (glycerol and antifreeze proteins). As body water declined to approximately 0.4 mg · mg^{-1} dry mass, the concomitant increase in solutes had the additional benefit of increasing viscosity and enhancing AFP activity leading to

deep supercooling. The low temperature observed, however, was not the lower limit of supercooling (Bennett *et al.* 2005). Supercooling points near -58 °C appeared to be a threshold below which no further evidence of freezing was observed: if individuals avoided freezing to at least -58 °C, no individuals were found to freeze when cooled to the -70s °C (the lower limit of the cooling bath). Under separate experimental procedures, it was shown that there was no detectable tendency to form ice found to -150 °C. The combination of low body water, the increase in viscosity, and low temperatures promoted vitrification of body fluids that augment survival of individuals well beyond minimum temperatures officially recorded in Alaska (Shulski and Wendler 2007).

Although some individuals collected at a certain place and time reach deep supercooling levels and can vitrify (mean glass transition temperature of -72 °C), not all larvae avoid freezing. In fact, differences were noted in deep supercooling status between *Cucujus clavipes puniceus* larvae collected from Wiseman and Fairbanks (see Chapter 2). Fairbanks and Wiseman, locations in interior Alaska, provide low temperature winter settings with which to examine extreme overwintering physiology of insects. Both locations have some of the coldest environments in North America. An official recording of -52 °C was noted in Fairbanks in 1962. In 1971 at Prospect Creek Camp 87 km south of Wiseman and approximately 107 km south of the northern limit of *C. c. puniceus*, -62 °C was recorded (Shulski and Wendler 2007). Wiseman larvae have the ability to deep supercool as early as November compared to only the beginning of December for Fairbanks individuals; and Wiseman larvae deep supercooled over a greater range of individual water content (0.3 to 0.9 mg · mg^{-1} dry mass), after they had

been winter acclimatized. One reason for these differences may have to do with environmental conditions. In Wiseman, low snow accumulation resulted in comparable above- and below- snow ambient temperatures, with minima near -30 °C (see Chapter 2). In contrast, in Fairbanks, snow cover typically resulted in a blanket of snow sufficient to buffer below-snow minima between -10 to -20 °C (for more detailed examinations of these temperatures in these two locations, see Wagener 1995; Bennett *et al.* 2005).

From previous work (Bennett *et al.* 2005), the authors speculated that low water content promoted deep supercooling in *Cucujus clavipes puniceus* larvae. We hypothesized that individuals with body water content $\leq$ 0.5 mg · mg^{-1} dry mass would deep supercool. To test this hypothesis, we evaluated the water content, the effects of ambient temperature one day prior to when insects were collected, and overwintering location on deep supercooling capacity in larvae from Wiseman and Fairbanks, Alaska, over a three year period. In addition, we used logistic regression to determine the probability of the dichotomous outcome–freezing or not freezing–to evaluate the relative contributions of these potential variables.

Methods

Insect Collection and Microhabitat Characteristics

We collected *C. c. puniceus* larvae from under the bark of fallen and standing dead Poplar spp. trees each September 2005–2007 near Fairbanks (64° 72' N) and Wiseman (67° 30' N), Alaska, a distance of approximately 437 km. Wiseman Alaska is 100 km north of the Arctic Circle; the latitudinal tree line is approximately 50 km north of Wiseman, and altitudinal treeline is about 150 meters high on local ridges; therefore,

this site is near the distribution limit for *C. c. puniceus*. After collection, larvae were placed in plastic food storage containers (20 x 15 x 10 cm; 20 –150 per box) perforated for gas exchange, along with moist bark from their host trees. Insects were left to acclimatize to local conditions by placing the containers either on the ground where they were likely to be covered by insulating snow cover or above the ground approximately two meters where they were un-insulated by snow. Larvae collected from poplar trees in Fairbanks were placed in a wooded area on the University of Alaska Fairbanks campus. Insects collected near Wiseman were held either at Wiseman or at the University of Alaska site. Air (ambient) and microhabitat temperatures at these overwintering locations were monitored using Hobo Pro Series data loggers and downloaded with BoxCar Pro 4 software (Onset Computer Corporation, Bourne, Massachusetts, USA).

Determination of Supercooling Points

Insects were recovered from containers at different times in winter and brought into the laboratory in Fairbanks to determine supercooling points and water content. Insects retrieved from Wiseman were kept at approximately -18 to -20 °C during transport to Fairbanks and tested within nine hours. Individual larvae were tested by placing a thermocouple junction (copper-constantan, 36 gauge) against the insect body in a 0.6 ml plastic tube. Thermocouple leads, up to 16, were attached to a computer controlled multi-channel thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, Ohio, USA) that recorded temperature in five second intervals. Closed tubes were placed inside a 500 ml glass beaker that was covered and mostly submerged in an alcohol-water cooling bath. Once insects equilibrated to 0 °C, bath

temperature was reduced at 0.2 °C/min, typically to -60 to -70 °C. The lowest body temperature recorded at the release of the latent heat of fusion, as evidenced by an exotherm, was recorded as the supercooling point. Larvae that did not exhibit an exotherm to < -58 °C were designated as deep supercooling.

Water Content

Individual larvae were weighed to the nearest 0.1 mg and then dried at 60 °C (48 h) to constant mass. Water content (WC) was calculated as mg · mg^{-1} dry mass (Rojas *et al.* 1986; Hadley 1994).

Probability of Freezing

Logistic regression models an event (freezing) occurring as a linear function of the predictor variables compared on a log scale. Odds are defined as a ratio of the probability of an event occurring over the probability of an event not occurring. From the logistic model, probabilities can be estimated. By exponentiating the parameter estimates, log (odds) can be transformed into odds and then into probability, where probability = odds / 1 + odds. An *a priori* set of potential predictors of whether freezing would occur and all appropriate two-way interactions were used to derive the model (Table 3.1). Non-significant interactions were dropped, and the reported results are for reduced models. Mean-b (mean temperature below-snow) was created by averaging microhabitat temperatures recorded at the collection site every 30 minutes for 24 hours prior to when animals were retrieved and brought to the laboratory to be tested for supercooling ability. Due to the distance between Wiseman and Fairbanks and the time

required for a single complete supercooling run, it was necessary to store larvae at -18 °C. This temperature was not figured into mean temperature one day prior to a SCP run.

Using the values from the parameter estimates of the reduced model, an LD50 can be estimated. Specifying water content as the principle parameter, LD50 estimates the water content at which 50 % of larvae freeze. The general equation is the following:

$$LD_{50} = 0.5 = e^{(\alpha + \sum \beta i Xi)} / 1 + e^{(\alpha + \sum \beta i Xi)}, \quad \text{eq. 1}$$

where α = intercept term, β_i are the β coefficients associated with statistically significant variables derived from the logistic regression analysis.

Logistic regression was performed with SAS (SAS 9.1, SAS Institute, Inc), and R (2.3.0, The R Foundation for Statistical Computing) was used to graph the probability of freezing in Fairbanks and Wiseman larvae. A Spearman Rank Correlation test was used *a priori* to estimate correlation between mean above-snow and mean below-snow temperatures. Unless otherwise noted, values are given as means, ± standard error of the mean, and number of individuals used to estimate mean (N): (mean ± S.E.M, N).

Results

Winter Microhabitat Characteristics

With an insulating cover of snow, minimum below-snow temperatures typically ranged between -10 to -15 °C, while above-snow temperatures exceeded -30 °C and often approached -40 °C and lower for various lengths of time, as presented in Figure 3.1. In January 2007 in Wiseman, the containers with insects placed on the ground were clearly visible due to the lack of snow fall. This low snow year resulted in similar above- and below-snow temperatures and lower minimum below-snow temperatures than typically

recorded; for example, on 7 January 2007, above-snow temperature was -43 °C and below-snow temperature was -40 °C (see Chapter 2). Above-snow minimum temperatures in Fairbanks and Wiseman were similar, ranging down into the -40s °C.

In Fairbanks and Wiseman, the Spearman Rank Correlation tests indicated that the mean above-snow and mean below-snow temperatures were correlated (Fairbanks: $\rho = 0.855$, P-value < 0.0001 N = 10896. Wiseman: $\rho = 0.837$, P-value < 0.0001, N = 10896); therefore, only mean-b (mean below-snow temperature for 24 hours prior to SCP determination) was chosen for further analysis since many *C. c. puniceus* were typically collected during winter months at or below snow level.

Supercooling, Deep Supercooling, and Water Content

Changes in SCP, WC, and ambient temperature over one overwintering season are presented (Fig. 3.1). For a more detailed examination, see Chapter 2). By late November 2006, mean SCP and WC were approximately -30 °C and < 0.9 mg · mg^{-1} dry mass. There were further decreases in both variables by January 2007, where mean SCP was near -50 °C and WC dropped to 0.5 mg · mg^{-1} dry mass. Note that these data do not include larvae that deep supercooled, i.e., did not freeze. As ambient temperatures decreased between summer and autumn, mean supercooling points determined in *Cucujus* larvae collected from Fairbanks and Wiseman populations declined by 20 °C from values as high as -7.0 °C and by a further 6 °C to 7 °C between autumn and winter to average seasonal minima of approximately -37 °C (Table 3.2). The lowest temperature that any larvae exhibited an exotherm (the lowest SCP) was -54.3 °C, regardless of

collection location. Larvae that supercooled to below -54.5 °C remained unfrozen to the lowest temperatures we were capable of testing. Wiseman larvae deep supercooled (no exotherm) when cooled to -70 °C (Table 3.2) as early as November, but it was not until December that Fairbanks larvae deep supercooled. Since winter values for mean SCPs and body water content of larvae collected in Wiseman and held in Wiseman or Fairbanks did not differ (Sformo *et al.* 2009), data were combined and are collectively referred to as larvae from Wiseman. Supercooling points and water contents within months were not statistically different (Table 3.2), except for the comparison between Fairbanks and Wiseman water contents in October - early December (Table 3.2).

Mean water content of frozen and supercooled larvae were significantly different, with deep supercooling larvae having between one-half and one-quarter the amount of water per milligram dry tissue as compared to larvae that froze. Mean water content (combined average) of individuals that deep supercooled was about 0.3 mg · mg^{-1} dry mass (Table 3.2).

Mean supercooling point temperatures, water contents, and percentage of insects that deep supercooled are shown in Figure 3.2 by location of collection and below-snow temperature during the 24 hours prior to collection. The range of WCs associated with high probabilities of deep supercooling in Fairbanks was 0.2 to 0.6 mg · mg^{-1} dry mass, and the range of below-snow temperature was -8.3 to -17.4 °C (Fig. 3.2). An increase in the proportion of deep supercooling in the Fairbanks population took place at WCs near 0.4 mg · mg^{-1} dry mass and below-snow temperatures near -11 °C (Fig. 3.2). In contrast, the Wiseman population displayed a more gradual increase in deep supercooling that

extended over a greater range of WCs and below-snow temperatures. WC varied between 0.3 to 0.9 mg · mg^{-1} dry mass, and the range of below-snow temperature was -3 to -20 °C (Fig. 3.2).

Log (odds), Probability of Freeing, and LD50

All main effects in the initial model were significant (not shown), and the Fairbanks population significantly differed ($P < 0.05$) from both Wiseman insects held in Fairbanks and from Wiseman insects held in Wiseman, but the latter two did not significantly differ from each other ($P = 0.5269$). This indicated that the original location of larvae rather than the overwintering location was important; therefore, we combined insects collected from Wiseman and re-ran the analysis with two populations (Fairbanks vs. Wiseman). This analysis revealed a significant interaction (Table 3.3) between water content and mean below-snow temperature (MB1) in predicting whether insects froze. The Goodness-of-Fit (Hosmer and Lemeshow) test indicated that the model fit the data: Chi-Sq: 3.646, df = 7, $P > 0.9$, and the C statistic was 0.96 out of 1.0, also indicating that the model fits the data.

Positive parameter estimates (β_is) (Table 3.3) indicate a linear increase in the log (odds) of freezing, whereas negative parameter estimates indicate a decrease in the log (odds) of freezing. For example, the negative parameter estimate of "loc" indicates that the Wiseman population is less likely to freeze than the Fairbanks population. The parameter estimate of water content indicates that for each increase of 0.1 mg · mg^{-1} dry mass, there is an 11.47-fold increase in log (odds) of freezing. Similarly, when below-snow temperature one day prior to testing (MB1) increased by 1 °C, there is a 0.44

increase in log (odds) of freezing. The significant interaction between water content and below-snow temperature indicates dependence of freezing upon both variables. Plotting the (log) odds of freezing by WC and MB1 (Fig. 3.3) reveals the interaction: larvae at higher temperatures have greater log (odds) of freezing than larvae at cooler temperatures, and larvae with greater WC do not have as great a decrease in the log (odds) of freezing as larvae with a lower water content.

By exponentiation, the log (odds) parameter estimates are transformed into probability (see methods), and by specifying 5 °C changes in MB1 and 0.1 mg · mg^{-1} changes WC, the probability of freezing in Fairbanks and Wiseman larvae is shown in Figure 3.4. In general, there is a greater decrease in the probability of freezing in Wiseman than in Fairbanks larvae for a given temperature (Temp) and water content (WC). In the upper figures (Fig. 3.4), the probability of freezing by WC at a specified temperature (colored lines) is shown in the typical "s-shaped" curves associated with logistic regression. At 0.6 WC, regardless of temperature, the probability of freezing varies between 93 to 98 % for Fairbanks larvae, whereas for Wiseman larvae probability of freezing varies between 82 to 95 %. At the lowest WCs, probability of freezing varies between 0 to 39 % for Fairbanks larvae, whereas for Wiseman larvae probability varies between 0 to 18 %. In Figure 3.4, the lower figures represent the decline in the probability of freezing for insects by temperature at given WCs (colored lines). In the lower figures, the dotted lines bracket the highest MB1 at which deep supercooling was observed that ranged for Fairbanks larvae between -8.3 to -17.4 °C and for Wiseman larvae between -3 to -20.1 °C. Taking 0.4 mg · mg^{-1} dry mass as expedient value of mass,

the probability of freezing for Fairbanks larvae varies approximately between 10 to 50 %, while probability of freezing for Wiseman larvae varies approximately between < 5 to 50 %.

Utilizing eq. 1 for LD_{50} and specifying WC as the principle parameter, we estimate LD50 by the following equation:

$$WC_{50} = (-\alpha - \beta_2 X_{loc} - \beta_3 X_{MB1}) / (\beta_1 + \beta_4 X_{MB1}), \quad \text{eq. 2}$$

Fairbanks larvae, as compared to Wiseman larvae, must lose more body water to reach 50 % of the larvae freezing (Table 3.4). Since there is a significant interaction between water content and temperature, the LD50 will be different at each temperature.

Discussion

The logistic regression analysis revealed that the probability of *C. c. puniceus* larvae freezing (and, therefore, dying) decreased with decreasing body water content and microhabitat temperature, measured over one day prior to sampling, in both populations. The probability (and log (odds)) of freezing declined at various "rates" (slopes in Fig 3.3) as a function of the interaction between temperature and water content. The general assessment of the probability of freezing is similar for both Wiseman and Fairbanks larvae: if temperature was held constant, then individuals with the highest water content had the highest probability of freezing, and individuals with the lowest water content had the lowest probability of freezing (Fig. 3.4, upper panels); if water content were held constant and individuals were subjected to increasingly lower temperatures, then the factor by which the probability of freezing declined was greatest for larvae with the lowest water content (Fig. 3.4, lower panels); however, the interaction between

temperature and water content on percent deep supercooling (Fig. 3.2) and LD50 (Table 3.4) differed between Fairbanks and Wiseman larvae.

The differences between the proportions of larvae that deep supercool in Wiseman vs. Fairbanks, especially at the warmer microhabitat temperatures, may reflect greater variation in environmental conditions. In Fairbanks, 90 % of individuals deep supercooled when temperatures were < -9 °C and WCs ranged from 0.2 to 0.5 mg · mg^{-1} dry mass (Fig. 3.2). Only two individuals (3 %) were found to deep supercool at temperatures above -9 °C: one deep supercooled with 0.6 mg · mg^{-1} dry mass and at MB1 of -8.9 °C, and the second deep supercooled with 0.4 mg · mg^{-1} dry mass and at MB1 of -8.3 °C. Using the 90 % criteria specified above, we not only found that 67 % of larvae from Wiseman deep supercooled within these ranges of temperature and WCs but also that 33 % deep supercooled at temperatures > -9 °C (up to -3 °C) and within a range of WC of 0.4 to 0.9 mg · mg^{-1} dry mass. While Fairbanks and Wiseman have similar low ambient (above-snow) temperatures, winter in Wiseman tends to commence earlier in the fall and last longer into the spring (Bennett *et al.* 2005). In addition, Sformo *et al.* (see Chapter 2) have shown that at least below-snow temperature in Wiseman on one occasion between 2002-2007 approached -30 °C in a low snow year. In Fairbanks, over this same time period, the lowest temperature recorded was approximately -23 °C. With the earlier onset and longer duration of below-snow temperatures in Wiseman, the differences could lead to the greater likelihood of Wiseman individuals deep supercooling due to the greater differential in vapor pressure of the frozen habitat and the vapor pressure of the supercooled body fluid, leading to increased water loss.

The loss of WC required to reach deep supercooling was less in the Wiseman larvae than in the Fairbanks larvae (Table 3.4), suggesting that the former may have lower water stress because Wiseman larvae can maintain greater WC and still deep supercool as well as reach 50 % probability of freezing at higher MB1 (Fig. 3.4, lower panels). During periods when larvae froze and others were capable of deep supercooling, water content of those that froze was 0.8 mg · mg^{-1} dry mass, and the mean supercooling point was approximately -37 °C, regardless of population. The temperature-dependent water contents found to deep supercool in Fairbanks larvae ranged between 0.2 to 0.6 mg · mg^{-1} dry mass (Fig. 3.2). The WC ratio between larvae that froze and larvae that deep supercooled from Fairbanks larvae represents a 2.0 to 4.0-fold further decrease in WC beyond the water already lost in late autumn–winter. In contrast, in the Wiseman population, individuals that deep supercooled had WCs ranging from 0.3 to 0.9 mg · mg^{-1} dry mass. The WC ratio between larvae that froze and larvae that deep supercooled from Wiseman represents only a 2.6-fold further decrease in WC beyond that already achieved. Theoretically, then, the low WC of Wiseman larvae that froze in December–March already approached WC necessary to deep supercool. Although this indicates that other variables may be important to deep supercooling, it also indicates a lower demand of further water loss (and possibly even permits a slight gain in WC); hence, there is less water stress in the Wiseman individuals. While these differences in WC between and within the location of these larvae might appear to be slight, it should be kept in mind that the lower water content in the Fairbanks population may be approaching a limit near 0.25 g · g^{-1} dry mass, a water content value associated with problems in the physical properties

of bio-molecules such as membrane fluidity (Crowe *et al.* 1988), although it should be kept in mind that Crowe *et al.* (1983) showed that some organisms are capable of surviving water losses below this value.

Moderate water loss and low temperatures, in addition to colligative antifreeze (glycerol) and noncolligative antifreeze proteins (Bennett *et al.* 2005; Sformo *et al.* 2009), allow larvae to avoid freezing under winter conditions in interior Alaska to approximately -40 °C (Table 2). Most larvae are found to overwinter under the bark of trees at or below snow cover; however, some individuals can be found to overwinter above snow where ambient temperatures are lower than -40 °C. Also, there are times when below-snow temperature is not moderated by sufficient snow cover, and larvae overwintering closer to the ground may experience temperatures more typical of above-snow conditions. The interaction between low microhabitat temperature and low water content allows individuals to deep supercool (< -58 °C). For these larvae, there is no evidence of freezing at temperatures. In fact, larvae enter a stable, non-crystalline vitreous state near -58 °C, with a mean glass transition (vitrification) temperature near -70 °C (see Chapter 2). Once larvae deep supercool to temperatures < -58 °C, they potentially undergo vitrification that should allow larvae to be stable for long periods of time due to the unique properties of a vitrified substance. Larvae in a vitrified condition will not have the stress of volume expansion and grain growth of ice in tissues (Hochachka and Somero, 2002); and since the vitrified condition encompasses both intra- and extra- cellular fluids, vitrified larvae should not encounter any differential osmotic and ionic stress (Storey, 2004) that is associated with concentration of solutes when

extra-cellular fluids freeze. This vitrified fluid is well-protected against freezing, showing no detectable tendency to form ice during transitions in temperature from -70 to -150 °C.

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Figures

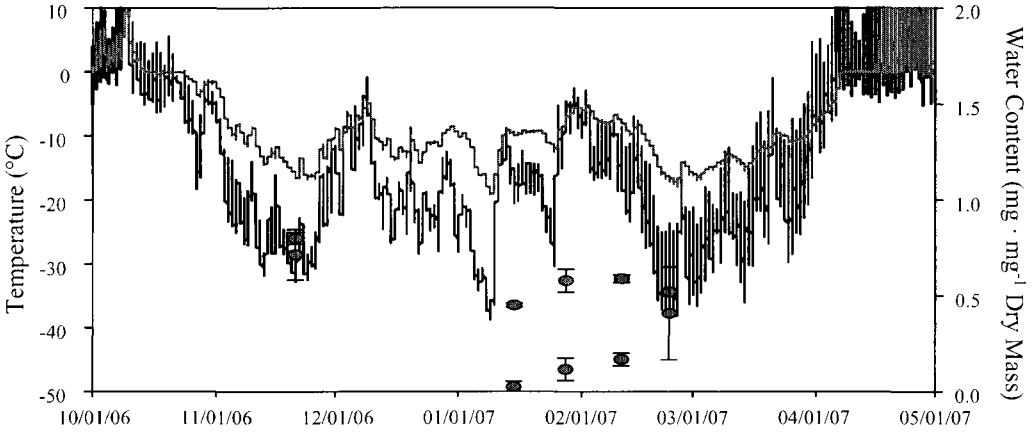

Figure 3.1 Above- and below- snow temperatures in Fairbanks and supercooling points and water content. Above- and below- snow temperatures (black and red lines, respectively) from Fairbanks, mean (± s.e.m.) supercooling points (blue ovals), and water content (red ovals) October to May 2006-07. Note that larvae on some of these dates deep supercooled (did not freeze), and these are not included in the data shown.

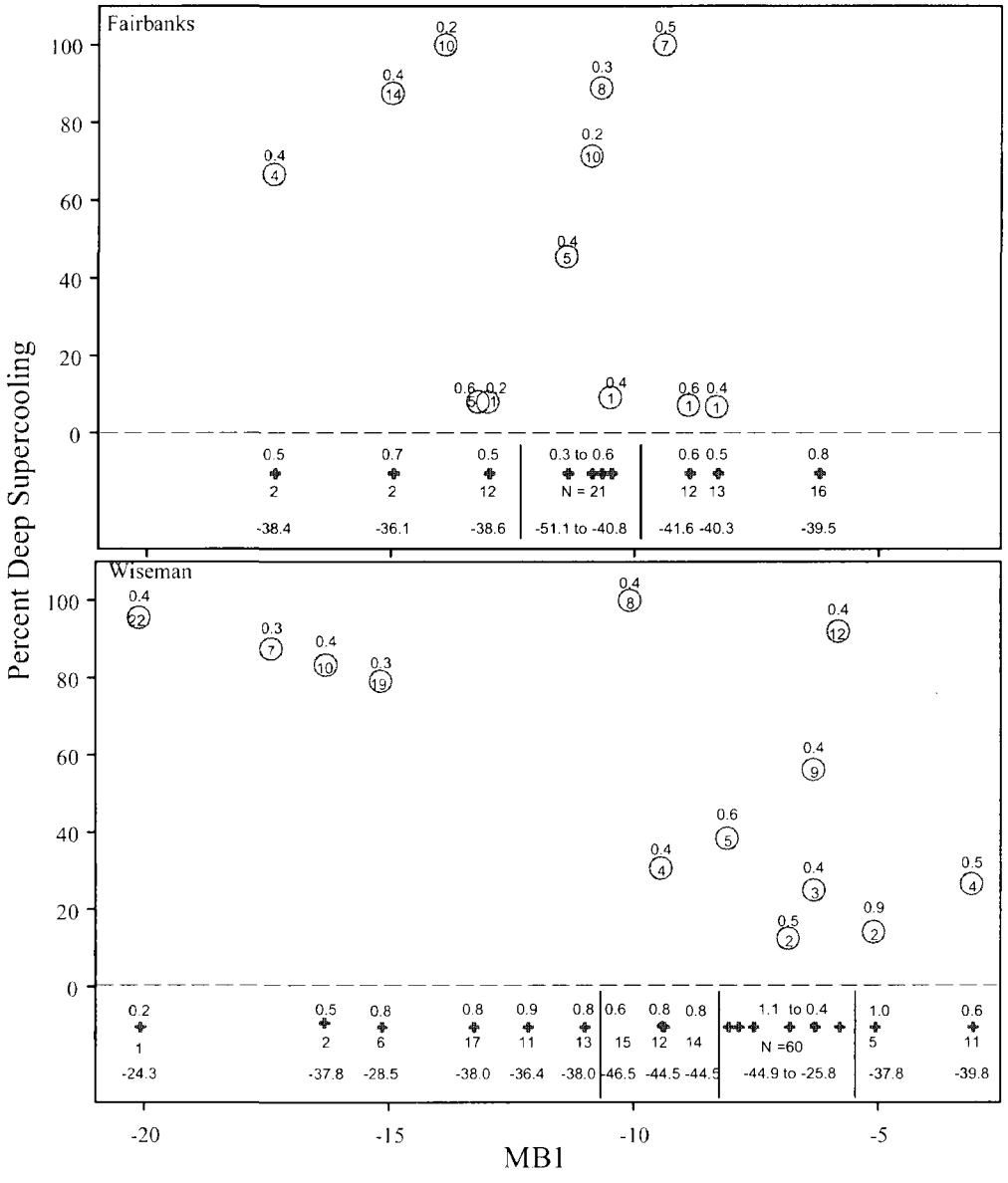

Figure 3.2 The percent of deep supercooling by below-snow temperature. The percent of deep supercooling by mean below-snow temperature 24 hours before testing (MB1, °C) is shown for Fairbanks and Wiseman larvae. Supercooling tests that resulted in individuals deep supercooling are designated above the dotted line (0 % deep supercooling) in red circles, and individuals trails that resulted in larvae that froze (supercooled) are displayed below the dotted line and with blue cross hairs. Above each shape is the mean value for water content (mg · mg^{-1} dry mass) per test. Number of individuals per supercooling test is shown within the circle or below the cross-hairs. Mean supercooling points (°C) per trial are shown below cross hairs. There is not necessarily a one-to-one correspondence between deep supercooling and supercooling trials since in each case some trials resulted in either 100% deep supercooling or 100% freezing. Some results overlapped MB1 and could not be displayed individually; therefore, the high and low minimum and maximum water contents and supercooling points and total number of individuals tested are shown grouped.

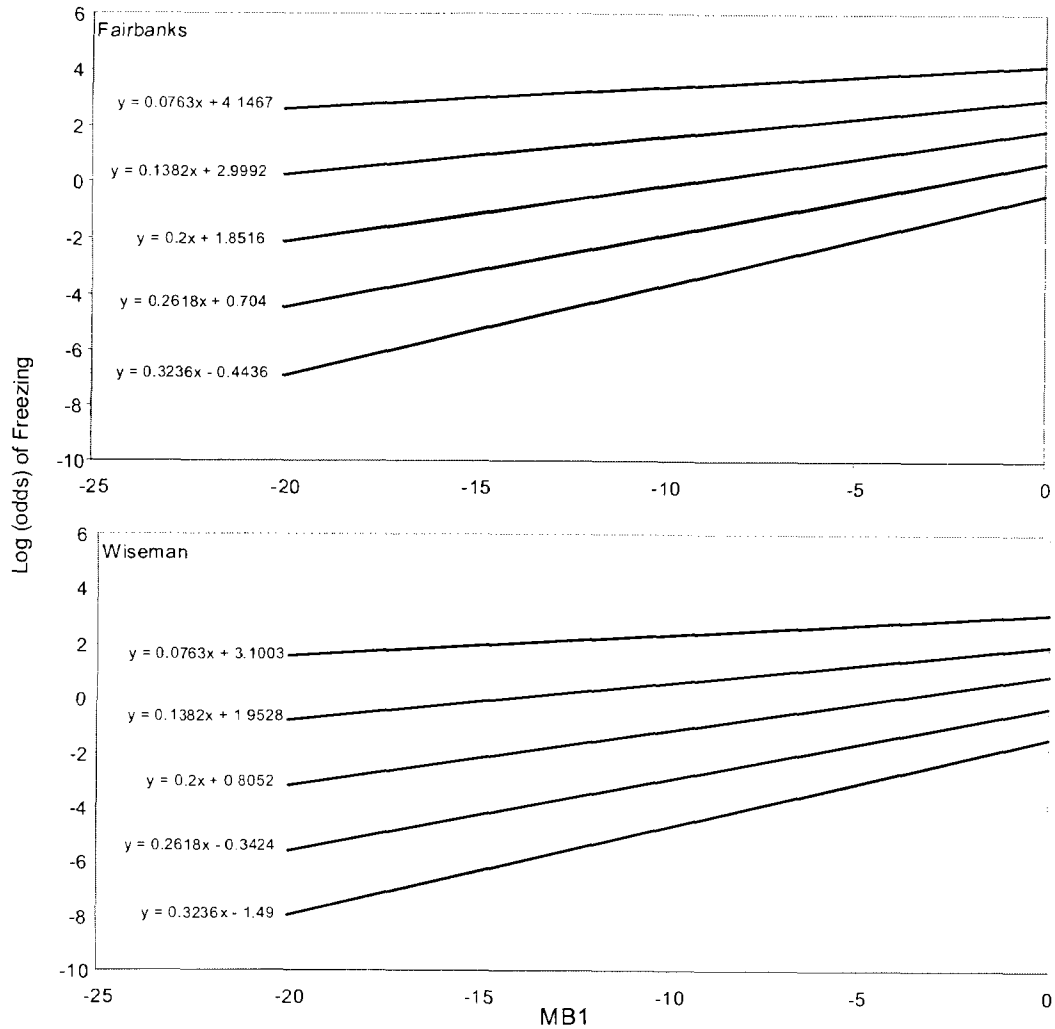

Figure 3.3 The log (odds) of freezing. The log (odds) of freezing by mean below-snow temperature (MB1) at selected water contents (WC, mg · mg^{-1} dry mass) specified by individuals curves from the first (top curve of each panel) from 0.6, 0.5, 0.4, 0.3, and 0.2 (lowest curve in each panel) mg · mg^{-1} dry mass.

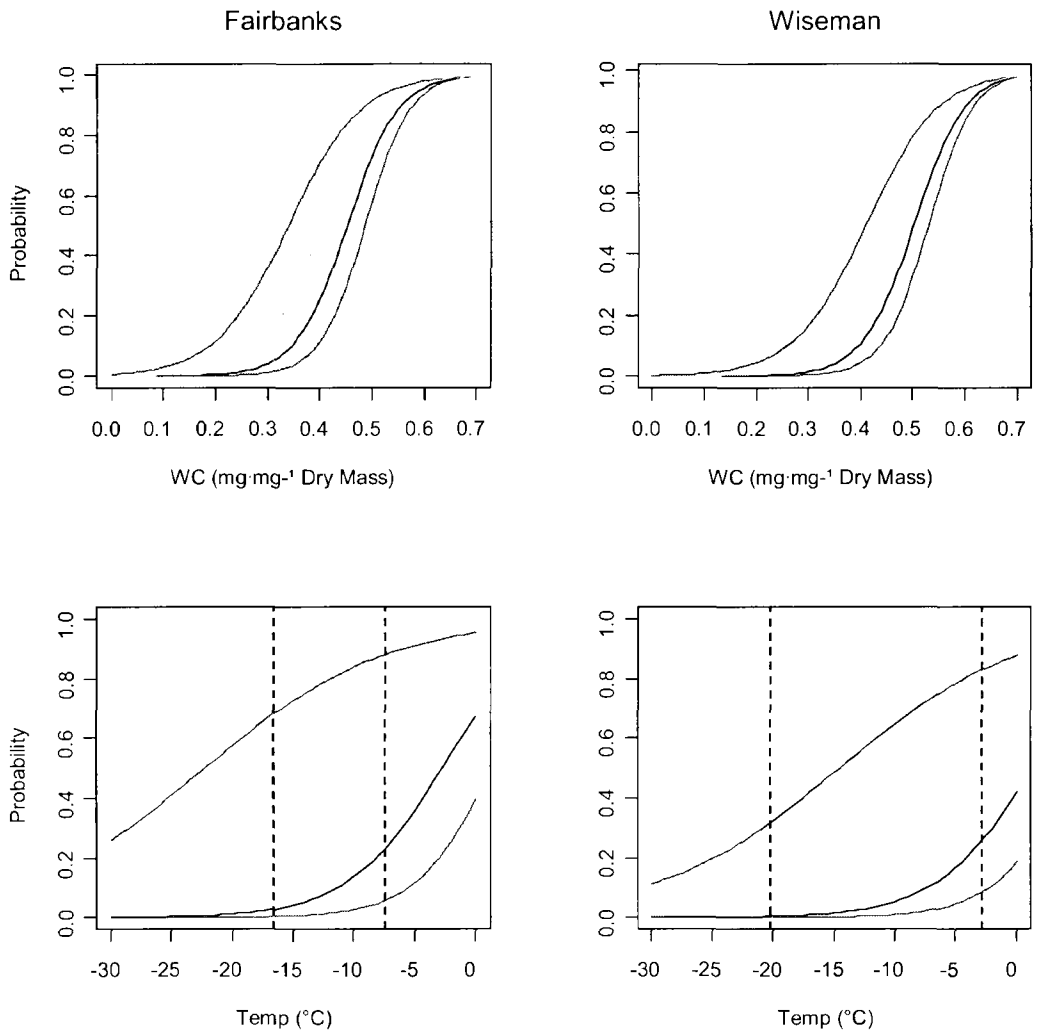

Figure 3.4 Interrelationships among probability of freezing of larvae from Fairbanks or Wiseman and insect water content (WC, mg · mg^{-1} dry mass) and temperature (Temp, °C) one day prior to testing. Since there is a significant interaction, each figure displays separate pieces of information: The vertical axis is probability that an individual will freeze, and the horizontal axis in the upper two figures is WC. The colored lines in the upper figures show the decline in the probability of freezing at WCs and at a given temperature (°C): cyan = temperature at 0, blue = -5, green = -10, black = -15, and red = -20. In the lower two figures, probability of freezing by temperature is displayed with the colored lines representing WC (mg · mg^{-1} dry mass): cyan = 0.6, blue = 0.5, green = 0.4, black = 0.3, and red = 0.2. Probability is extrapolated at WC < 0.2 and > 0.6 and temperatures > -5 and < -20. The vertical dotted lines approximate the range of MB1 when larvae were actually observed to deep supercool.

Tables

Table 3.1 List of the potential variables for the logistic regression model. A list of the potential variables (main effects and two-way interactions) for the logistic regression model of larvae freezing vs. not freezing.

Null	Random outcome	$\log[p/(1-p)]= \beta_0$
WC	Water content (mg · mg^{-1} dry mass)	$\log[p/(1-p)]= \beta_0 +\beta_1 WC$
Location	Population@Location	$\log[p/(1-p)]= \beta_0 +\beta_1 Loc$
Mean-b (below-snow)	Mean below-snow temperature (°C) one day prior to SCP run	$\log[p/(1-p)]= \beta_0 +\beta_1 Mean\text{-}b$
WC | Location	Water content | Loc	$\log[p/(1-p)]= \beta_0 +\beta_1 WC +\beta_2 Loc$
WC | Mean-b	Water content | Mean-b	$\log[p/(1-p)]= \beta_0 +\beta_1 WC +\beta_4 Mean\text{-}b$

Note: " | " designates interaction

Table 3.2 Seasonal changes in supercooling and water content of insects that froze vs. those that did not freeze. Seasonal changes in mean (±SEM (N)) supercooling (°C) and water content (mg · mg^{-1} dry mass) of insects that froze (exotherm) and did not freeze (no exotherm to -70 °C).

Collection Months	Location of Population	Supercooling Point	Water Content (insects that froze)	Water Content (insects that deep supercooled)
Oct.-early Dec.	Fairbanks	$-28.2^a \pm 1$ (65)	$1.4^a \pm 0.05$ (65)	NA
Oct.-Nov.	Wiseman	$-30.0^a \pm 1$ (91)	$1.2^b \pm 0.04$ (91)	$0.3^d \pm 0.03$ (32)
Late Dec.-Apr.	Fairbanks	$-36.4^b \pm 0.7$ (174)	$0.8^c \pm 0.03$ (175)	$0.3^d \pm 0.02$ (52)
Dec.-March	Wiseman	$-38.7^b \pm 0.8$ (168)	$0.8^c \pm 0.02$ (170)	$0.4^d \pm 0.02$ (59)

Superscript letters indicate significant difference in means ($P < 0.05$) with Tukey-Kramer adjustment for multiple comparisons.
NA = not available.

Table 3.3 The reduced logistic regression model. Results for the reduced logistic regression model specified for two locations: Wiseman larvae are composed of individuals collected in Wiseman and held in Wiseman or Fairbanks; Fairbanks larvae were collected and held in Fairbanks.

Parameter	DF	Estimate	STD Error	Wald Chi-Square	Pr = ChiSq
Intercept (α)	1	-2.73	1.18	5.30	< 0.0213
WC (0.1 mg·mg^{-1}) (β_1)	1	11.47	1.87	37.34	< 0.0001
loc Wiseman (β_2)	1	-1.04	0.37	7.85	0.0051
mb1 (1°C) (β_3)	1	0.44	0.08	26.70	<0.0001
WC × mb1 (1°C) (β_4)	1	-0.61	0.14	18.95	<0.0001

Abbreviations:
WC = water content, loc = location, mb1= mean below-snow temperature one day prior to testing

Table 3.4 LD50 estimates of water content. From the model, estimated water content (WC, mg · mg^{-1} dry mass) by location at which 50 % of insects froze (WC50) calculated at various temperatures (°C) by eq. 2.

	Temperature				
Population	-20	-15	-10	-5	0
Fairbanks (WC)	0.49	0.45	0.40	0.34	0.23
Wiseman (WC)	0.53	0.50	0.46	0.41	0.32

Chapter 4 Simultaneous freeze tolerance and avoidance in individual fungus gnats, *Exechia nugatoria*[1]

Abstract

Freeze tolerance and freeze avoidance are usually described as mutually exclusive strategies for overwintering in animals. Here we describe an insect species that combines both strategies. Individual fungus gnats, collected in Fairbanks, Alaska, display two freezing events when experimentally cooled and different rates of survival after each event (mean ± s.e.m.: -31.5 ± 0.2 °C; 70 % survival and -50.7 ± 0.4 °C; 0 % survival). To determine what body compartments froze at each event, we dissected the abdomen from the head/thorax and cooled each part separately. There was a significant difference between temperature levels of abdominal freezing (-30.1 ± 1.1 °C) and head/thorax freezing (-48.7 ± 1.3 °C). We suggest that freezing is initially restricted to one body compartment by regional dehydration in the head/thorax that prevents inoculative freezing between the freeze-tolerant abdomen (71.0 ± 0.8 % water) and the supercooled and freeze-sensitive thorax and head (46.6 ± 0.8 % water).

Key Words: Mycetophilidae, *Exechia nugatoria*, supercooling, exotherm

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[1] Sformo T, F. Kohl, J. McIntyre, P. Kerr, J. Duman, and B. M. Barnes 2009. Simultaneous freeze tolerance and avoidance in individual fungus gnats, *Exechia nugatoria*. Published in Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 179: 897-902 (DOI: 10.1007/s00360-009-0369-x) online June 04, 2009

Introduction

The fungus gnat *Exechia nugatoria* Johannsen (1912) (Diptera: Mycetophilidae) found in Fairbanks, Alaska, survives the winter as an adult above the snow under the bark of dead trees, a microhabitat that does not significantly insulate individuals from fluctuating ambient conditions, including low temperatures that can decline below -50 °C. Fungus gnats are a diverse and abundant group of insects that can be recognized by their hump-backed appearance, stout and elongate coxae, and well-developed tibial spurs. They often attain large population sizes and play an important role in the food web within the forest environments where they reside. Mycetophilidae are the most diverse in the northern Palearctic Region (Jaklovlev and Siitonen 2004), and in cold climates many members are known to overwinter as adults, particularly those of the tribe Exechiini. Their overwintering sites include caves (Kjaerandsen 1993; Kurina 1996; Hedmark 2000), hollow stems of umbelliferous plants (Väïsänen 1981), and under the bark of dead trees (Hedmark 2000). In these studies, microhabitat temperatures were found to be relatively moderate (approximately -6 °C).

In extreme cold climates such as the interior of Alaska, insect species either avoid or tolerate freezing to survive winter. Insects may behaviorally seek microhabitats that ameliorate low subzero temperature. Freeze-avoiding species cease feeding and eliminate the contents of their gut to promote supercooling of body fluids to temperatures well below their equilibrium freezing point (Zachariassen 1985). In addition, these insects must remove ice-nucleating factors, prevent inoculative freezing by production of

noncolligative antifreeze proteins (AFPs) (Duman 2001), and/or synthesize colligative antifreezes such as glycerol and other polyols (Storey and Storey 1991). In contrast, freeze-tolerant insects withstand freezing of extracellular water, often by inducing freezing at high subzero temperatures, typically -5 to -12 °C, via ice-nucleating factors (Zachariassen 1985; Duman 2001); furthermore, in Alaska some freeze-tolerant insects can be found in microhabitats such as on vegetation that lies above the snow where exposure to low ambient conditions is relatively unimpeded (Miller 1982). Consequently, these individuals are often exposed to temperatures that can cause ice nucleation. In studies that included fungus gnats in Alaska, Miller (1978, 1982) showed that *Mycetophila* spp. and *Exechia* spp. in Fairbanks are freeze tolerant but supercool to -30 °C, a low temperature that is more typically associated with freeze-avoiding insects. He did not note dual freezing events in these gnats. He did describe two nucleation events occurring in a Lepidopteran (*Martyrhilda ciniflonella*, at -25 and -36 °C) and a Neuropteran (*Hemerobius simulans*, at -15 and -36 °C), although he did not report on differential survival of individuals between the two freezing events.

We present evidence that freeze tolerance and freeze avoidance in individual *E. nugatoria* are not mutually exclusive. We report on adults that survive initial freezing near -30 °C but do not survive a second freezing event near -50 °C. Understanding how these flies survive low temperature conditions may help explain how they have become widespread in cold regions.

Materials and Methods

Insect collection and microhabitat characteristics

We collected adult *E. nugatoria* November 2007–December 2007 from large aggregates of fungus gnats found under bark of dead and dry standing *Poplar* spp. in Fairbanks, Alaska, near the Fairbanks International Airport. We estimate that some trees housed greater than 1,000 individuals. All insects were located above the snow. Insects were transported in cold insulated containers and tested in the laboratory for freezing responses either within one hour of collection or after ten days of acclimation at 4 °C in 100 % relative humidity (RH).

On the north side of a tree at the collecting site, temperatures of the air (2.2 m above ground) and microhabitat (under the bark at 1 m above ground) were monitored using Hobo Pro Series data loggers and downloaded with BoxCar Pro 4 software (Onset Computer Corporation, Bourne, Massachusetts, USA).

Supercooling

To determine supercooling points of gnats, a single copper-constantan thermocouple junction (36# g) was placed against an individual. The junction and insect were placed in closed 0.6-ml plastic vials submersed in alcohol/water baths that were cooled from 0 °C at 0.2 °C/min. Thermocouple leads were attached to a computer controlled multi-channel thermocouple thermometer (Iso-Thermex, Columbus Instruments, Columbus, Ohio, USA) that recorded temperature every five seconds. The lowest body temperature recorded at the onset of freezing, as evidenced by an exotherm

(a transient rise in temperature due to the release of the latent heat of fusion), is the supercooling point (SCP).

Since we found that these insects displayed dual exotherms, we investigated which body compartments were responsible for each freezing event by dissecting with scissors the abdomen from the head/thorax (including wings, legs, and antennae), and cooling each body compartment in separate tubes. If either halter, the reduced second pair of wings used for balance, was accidentally severed, a new individual was used. Immediately after dissection, each body part was placed in a tube so that it rested against a thermocouple; a microscope was used to ensure the body part was in contact with the thermocouple junction. Since this visual inspection showed little to no fluid loss, the dissected parts were not sealed with oil.

To ensure that the second exotherm in whole insects was not dependent on the first, a supercooling run was performed in March 2008 in which the bath was programmed to hold at temperatures below the first supercooling point (approximately -34 °C) for 48 h. After 48 hours, the bath was programmed to cool to -60 °C to record a possible second exotherm.

Survival

To assess immediate survival of gnats removed from the field, insects were collected on several occasions in November and December 2007. They were brought to the lab and placed on moist towels at 4 °C and 100 % RH to assess survival (return of coordinated movements) over a one-week period. To determine whether flies survived the first freezing event, they were cooled to approximately -35 °C on 20 and 30

November and on 18 and 21 December 2007 and then warmed to 0 °C. Both cooling and warming rates were conducted at 0.2 °C/minute. Individuals were held overnight on ice. They were then transferred to a container at 4 °C and 100 % RH. Similarly, to determine whether flies survived the second freezing event, they were cooled to approximately -58 °C on 20 and 30 November 2007 then re-warmed as above.

Water Content

Masses of individual larvae (whole body) were determined to the nearest 0.1 mg. Larvae were dried at 60 °C for 5 days to constant mass. Absolute body water was calculated as mg · mg^{-1} dry mass and termed water content (WC) (Hadley 1994). Water contents of dissected body parts (the abdomen vs. the head/thorax) were similarly determined to the nearest 0.01 mg.

Results

Microhabitat temperatures varied widely from transient highs above 0 °C to lows of -41 °C during this study, and air and under-bark microhabitat temperatures were similar (Fig. 1; mean difference = 0.2 °C; n = 8281). From 1 December 2007 to 28 March 2008, mean temperature was -18.1 ± 0.1 °C (n=5710, recorded every 30 min).

Directly after collection from the field, gnats (fresh mass = 1.7 ± 0.4 mg, n=78) froze at an initial supercooling point (SCP1) of -31.5 ± 0.2 °C (Fig 2a); 70.2 % of these were alive after thawing, a survival rate similar to that in field-collected insects that were assessed for movement after direct transfer to the lab and warmed to 4 °C (Table 1;

combined average 85 %). Gnats cooled below SCP1 showed a second exotherm (SCP2) at -50.7 ± 0.4 °C (Fig 2a) that was lethal (Table 1).

The dissected body compartments of newly collected individuals were cooled as above to determine which body segments were responsible for SCP1 and SCP2. Supercooling point values of dissected gnat abdomens averaged -30.1 ± 1.1 °C and were statistically indistinguishable from average SCP1 values from intact gnats (Fig 2a). Supercooling values of dissected head/ thoraces were -48.6 ± 1.3 °C and did not differ from the average SCP2 in intact insects (Fig. 2a). These results suggest that freezing of the abdomen is responsible for SCP1, and freezing of the head/thorax complex is responsible for SCP2 in intact gnats. After 10 days of acclimation at 4 °C and 100 % RH, whole gnats froze at -20.6 ± 0.6 °C, and all died (Table 1). A second exotherm was not observed in these acclimated animals when they were cooled to -60 °C (Fig 2a).

Insects in the field are regularly exposed to temperatures below the SCP1 (Fig. 1). For example, insects were collected after a cold period 3-9 February 2008 (Fig. 1) when mean ambient temperature was -39.2 ± 0.1 °C (n = 282), with extremes of -31 and -43 °C. Survivorship was 91 % (64/70) when insects were brought into the laboratory on 18 February. This suggests that fungus gnats naturally survive the freezing of their abdomens and that, since freezing of the thorax and head is lethal, abdominal ice does not inoculate freezing in anterior body compartments, even when exposed to temperatures lower than SCP1 for several consecutive days.

We also demonstrated that SCP2 is not dependent on SCP1. On 28 March 2008, the bath was programmed to hold at approximately -34.5 °C for 48 hours. After this

hold, the bath was decreased to -60 °C to trigger SCP2. Although gnats appeared to be acclimating to spring conditions as early as 23 March and SCP2 increased to -42 °C ± 1.3 (N = 16), there was a statistically significant difference ($P< 0.05$; T-test with equal variance, $t = 5.50$, $P = < 0.001$, df = 24) in SCP1 vs. SCP2 (-33.2 ± 0.6 °C, n = 16 vs. -39.2 ± 0.8 °C, n = 10, respectively) with 10/16 individuals exhibiting SCP2. We believe that if this experiment had been carried out when gnats were still under winter field conditions, SCP2 would have been lower, and all would have displayed a second exotherm.

Discussion

Exechia nugatoria in winter displayed two freezing events when experimentally cooled; they survived the first that occurs at temperatures of approximately -32 °C, but they did not survive the second freezing event that occurred at approximately -51 °C. Testing body compartments separately suggests that it is the abdomen that freezes first while the head/thorax freezes second. Since fungus gnats overwintering in Alaska routinely survive temperatures between -30 and -40 °C, they must in nature become partially frozen and prevent the inoculation of ice from occurring between adjoining body segments.

Why did this unusual dual strategy evolve in this species? Lundheim and Zachariassen (1993) and Zachariassen *et al.* (2004, 2008) suggest that some freeze-tolerant insects that have greater trans-cuticular water permeabilities than related freeze-avoiding species conserve body water and avoid dehydration while frozen because they are in vapor pressure equilibrium with ice in the surrounding environment. Since the

overwintering site of *E. nugatoria* is a cold, dry microhabitat, an advantage of maintaining ice in the abdomen may be to lessen evaporative water loss over the course of a long winter. Not only would abdominal water be conserved but also some head/thoracic water vapor may be directed to the abdomen, rather than being lost to the environment. If the entire body froze, however, water loss would be even less. Freezing of the head and thorax may be avoided due to an increased susceptibility of neural tissue to damage from freezing as has been suggested to occur in other freeze-tolerant insects (Collins *et al.* 1997; Yi and Lee 2003). The large changes in ion concentrations in the remaining unfrozen fraction that result from extracellular freezing may not be tolerated by certain neurons or other cells of the anterior central nervous system.

A second obvious question arising from this study is, how is it possible for the head/thorax to remain unfrozen while water in the thorax is apparently in direct contact with the frozen abdomen? Freshly dissected abdomens contained approximately 71 % water, and heads/thoraces had 47 % water, due either to proportional differences in hard body parts such as cuticle in these different body compartments or the selective withdrawal of water from the thorax during freezing. Dehydration decreases SCPs in freeze-avoiding insects (Zachariassen 1985; Bennett *et al.* 2005), and resistance to inoculative freezing increases with dehydration in the Antarctic midge *Belgica antarctica* (Elnitsky *et al.* 2008); both of these relationships could contribute to the stability of partial freezing in the fungus gnat. While survival of an individual that both tolerates and avoids freezing has not been noted previously in insects, it occurs in plants, although the underlying mechanisms are not satisfactorily understood (Wisniewski 1995; Quamme

1995). Two exotherms are common in the stems of many species of trees and typically consist of a high temperature exotherm (HTE) between -7 to –13 °C and a low temperature exotherm (LTE) below –40 °C (Quamme *et al.* 1972; George *et al.* 1974). In stems of hardwood and softwood trees from temperate regions, the HTE is due to freezing of extracellular water, especially in the xylem, while the LTE represents freezing in freeze-sensitive xylem ray parenchyma. Consequently, avoidance of the LTE in the freeze-sensitive parenchymal tissue permits adaptation to relatively low temperatures in these trees but also imposes limits in their ranges in terms of latitude and extreme low temperatures (George *et al.* 1974) and altitude (Becwar *et al.* 1981). Water associated with the xylem ray parenchyma may remain supercooled while surrounding water in the xylem is frozen. This is due, at least partially, to cell wall structures and pectin interactions with the cell wall (Wisniewski 1995). Recently, freeze-avoiding xylem ray parenchyma of the katsura tree *Cercidophyllum japonicum* are reported to have flavonol glycosides with anti-ice nucleation activity that promote supercooling (Katsuga *et al.* 2008). Supercooling of freeze sensitive flower and leaf buds that are surrounded by frozen extracellular water is also common in trees (Quamme 1995). In conifers, after the extracellular water in tolerant tissues freezes, primordial shoot water migrates toward the frozen tissue where it subsequently freezes. Sakai (1979) describes this as 'extraorgan freezing' that allows the freeze-intolerant primordium to supercool to lower temperatures due to the removal of water. Cary (1985), working on *Prunis* (peach and prune) flowers, modeled supercooling in floral tissue with ice present, showing that water vapor flowing toward ice crystals may "cause a discontinuity in the liquid phase" that acts as a barrier to

nucleation. The abdominal freezing of the fungus gnat may function as "extraorgan freezing" allowing water to be withdrawn from the thorax to further decrease the supercooling point. In fungus gnats, abdominal freezing (-30 °C) and a subsequent movement of water vapor from the head/thorax may create a dry area (discontinuity) as a barrier against inoculative freezing of the remaining supercooled fluid in the thorax. This mechanism is dependent on a freezing event, yet our observations of water content differences were on unfrozen insects. These may have previously frozen and the differences in water content remained after thawing. Even though the gnats display a difference in water content after they have rewarmed, the gnat acclimation to above-freezing temperature and humidity after 10 days increases total body water through rehydration of the head/thorax that also results in high, lethal SCPs and the absence of SCP2. With the slight but significant increase in whole-body water content, there is an increase in the temperature of the first freezing event and elimination of the second, and no gnat survives.

This study presents a unique example of a mixed overwintering strategy in a single insect, tolerating freezing in the abdomen while avoiding freezing in the thorax and head. While a similar mixed overwintering strategy is more typical in plants, this is the first arthropod known to overwinter in this manner. At present, the mechanism(s) underlying this strategy and rationale for its evolution remain unknown.

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Figures

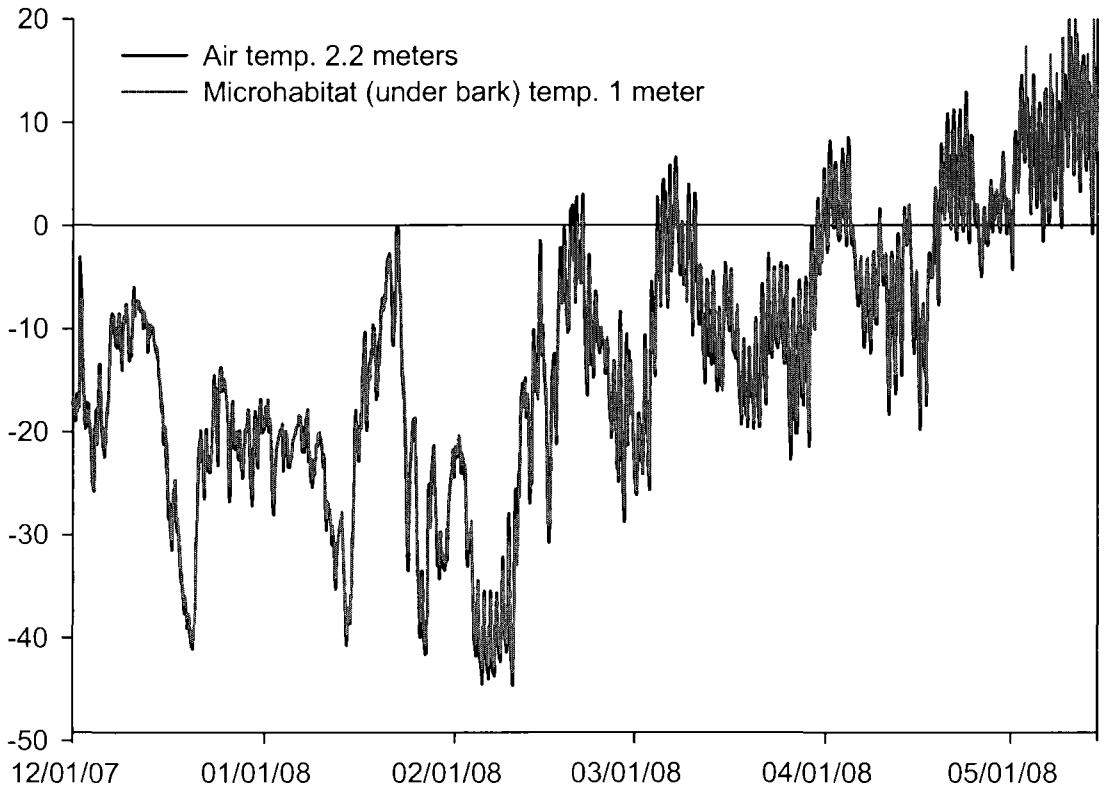

Figure 4.1 Microhabitat (under bark) temperature 1 meter above the ground (red) and air temperature 2.2 meters above the ground (black) at the fungus gnat collecting site.

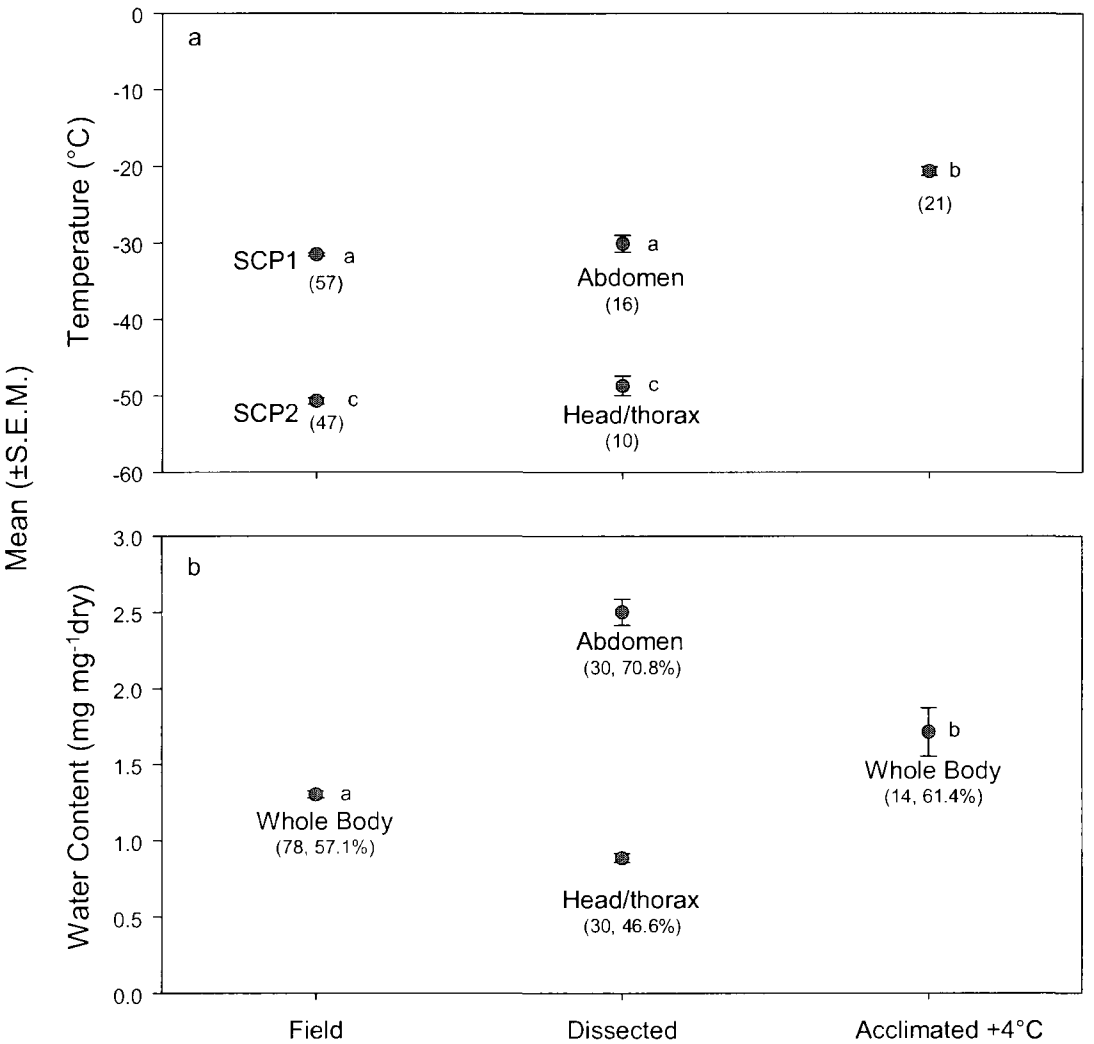

Figure 4.2 Mean (± sem) supercooling (a) and water content (b) in fungus gnats brought in from the field or after exposure to 4°C for 10 days. Numbers within parentheses are percent water and/or sampling number. Different letters indicate significant differences in means. In Figure 4.2a, SCPs of fungus gnats immediately after they were collected and transferred to the laboratory: Shapiro-Wilk normality test, $P < 0.05$; Wilcoxon two-sample test, $S=1653.0$, $P < 0.0001$. Abdomen vs. head/thorax is significantly different: Shapiro-Wilk test, $P > 0.05$; T-test with unequal variance, $t = -17.60$, $P = 0.0001$, $df=35.6$. SCP1 (collected vs. dissected): Shapiro-Wilk normality test, $P < 0.05$; Wilcoxon rank-sum, $S = 666.0$, $P = 0.3301$. SCP2 (collected vs. dissected): Shapiro-Wilk normality test, $P < 0.05$; Wilcoxon rank-sum, $S = 355.0$, $P = 0.1813$. In Figure 4.22b, whole body vs. whole body (acclimated) water content, Shapiro-Wilk normality test, $P< 0.05$; Wilcoxon rank-sum, $S = 1049.0$, $P < 0.0001$. For body compartments, Shapiro-Wilk test, $P < 0.05$; Wilcoxon rank-sum, $S = 465$, $P < 0.0001$.

Table

Table 4.1 Survival results based on individuals brought in from the field and experimentally frozen to SCP1 or SCP2 or after acclimation.

Date	Condition	Percent	N
11/16/07	Field	93.2	44
11/20/07	SCP1	81.3	16
11/20/07	SCP2	0.0	16
11/30/07	Field	92.1	126
11/30/07	SCP2	0.0	15
12/18/07	Field	68.5	73
12/18/07	SCP1	46.7	15
12/22/07	SCP1	81.3	16
12/28/07	SCP2	0.0	16
12/31/07	Acclimated	0.0	32
02/28/08	Field	91.4	70

*Condition:
Field = collected and immediately placed into 4 °C and 100 % RH; no supercooling.
SCP1 = supercooled below SCP1 to -34 °C.
SCP2 = supercooled below SCP2 to -58 °C.
Acclimated = collected and stored at 4 °C and 100 % RH for ten days, and tested acclimated individuals to a bath temperature of -29 °C, resulting in a mean SCP of -19.3 °C ± 0.9; none survived.

Appendices

These appendices provide future directions of my work on overwintering physiology in the form of a proposal (Appendix 1) and a summary on a variety of insects that I worked on during the dissertation but that were not examined in enough detail to warrant a full paper (Appendix 2). This research was conducted opportunistically or was conducted for others outside the Barnes/Duman collaboration. Appendix 3 provides raw data on the carpenter ant *Camponotus herculeanus*, and it is hoped that others will be able to utilize them for further overwintering work.

Appendix 1: Future Directions

My future directions include the study of *in situ* ice formation in floral and faunal tissues using a phase-enhanced synchrotron x-ray imaging system such as the one at the Argonne National Lab. All flora and fauna subjected to subzero temperatures face potential lethal freezing events, whether due exclusively to ice growth or associated increases in ion concentration (Zachariassen 1985; Duman 2001; Costanzo *et al.* 1995). In fact, in some freeze-tolerant organisms, the freezing leads to controlled hyperglycemia (Costanzo *et al.* 1995). The fate of tissue, therefore, in a freezing environment is complex, and the direct, internal visualization of these events will allow us to understand how diverse organisms tolerate ice and mitigate cryoinjury. Substantial economic and material losses due to uncontrolled freezing in cultivated agriculture products

(Tiefenbacher *et al.* 2000) and in tissue and organ preservation (Costanzo *et al.* 1995) can be ameliorated.

The advanced imaging I propose would provide direct internal visualization of ice formation in real time in distinct body compartments. Previous imaging work on freezing relies on either dissection or on external visualization such as infrared thermography. While these techniques provide insight, the fine structures of tissue must be destroyed in the former, while the latter relies on the surface effect of the release of the latent heat of fusion. At a cellular and tissue level, we do not know how ice growth is managed. In fact, we do not know whether bacteria that are "tolerant" of freezing are freeze tolerant as described in this dissertation or whether they are freeze avoiding. What actually freezes in a single-celled organism? Might ice form outside the cell, or does intra-cellular ice form in these organisms?

By using a phase-enhanced synchrotron x-ray imaging system with a high-speed video recording camera, I propose to image ice formation and mechanisms of inhibiting low-temperature ice propagation in diverse biological tissues. Although plant and insect anatomies differ, the biomechanical processes of ice formation, segregation, and the consequent movement of water may be similar.

Flora: The geographic distribution of woody plants has been correlated to survival of high temperature freezing events or exotherms (HTEs, ~ -11°C) and avoidance of low temperature exotherms (LTEs, ~ -40°C) in the primary water conducting tissue of plants (xylem) (George *et al.* 1974; Cary 1985; Wisniewski 1995; Quamme 1995). The movement of water and growth of ice during these exotherms has not been established

due to limited imaging techniques, yet knowledge of how and where ice is compartmentalized during freezing is essential to understanding freeze tolerance in many cultivated fruit crops (George *et al.* 1974; Sakai 1979; Cary 1985; Quamme 1995; Wisniewski 1995). Direct visualization of water, ice, and ice growth in intact xylem during HTEs and LTEs would answer this question. By examining angiosperms and gymnosperms from both eastern deciduous and boreal forests from three temperature zones (plants in areas where there is little probability of temperatures at or below -40°C, southwest USA; plants in areas with a greater probability of reaching this temperature, upper Midwest USA; and plants in areas where average minimums are below -40°C, northwest USA such as Alaska), I plan not only to examine the particularities of water/ice migration within plants but also correlate plant distribution on a cellular scale to water migration patterns within xylem (George *et al.* 1974).

Fauna: In contrast to woody plants, the geographic distribution of insects based on overwintering strategies is more complicated since diverse species at all stages of development can be found in the same location. The overlap in distribution, however, means that a great number of the major insect orders (Coleoptera (beetles), Diptera (flies), Hemiptera (true bugs), Hymenoptera (bees and ants)) can be surveyed from the same geographical and temperature related regions as described above. Movement of water and growth of ice during nucleation are critical to understanding the mechanisms of the overwintering strategies: What allows, for instance, freeze-tolerant insects, as opposed to freeze-avoiding insects, to survive ice formation? Anatomically, all insects have an open circulatory system, so a freezing event in one body segment would be

expected to inoculate the next. Freeze-tolerant insects tend to have HTEs (similar in temperature range to plants) that appear to nucleate a significant proportion of water throughout the body, as compared to single lethal LTEs (occurring at a wider range of temperatures than plants) in freeze-avoiding insects (Zachariassen 1985; Duman JG 2001). For insects that are simultaneously freeze tolerant and avoiding, like plants, how and where is ice propagation halted? Answers to these questions are compounded by the presence of three or four different extracellular fluid compartments that extend throughout the body of insects: hemolymph/heart, gut, extracellular fluid in the ventral nerve cord, and tracheoles. The phase-enhanced synchrotron x-ray imaging system will provide visualization of ice formation in live animals and within-body compartments. Direct internal imaging of ice will allow me to observe whether anatomical features such as air sacs inhibit ice growth and/or whether ice is inhibited near central nervous systems where increasing ion concentration could impede action potentials in neurons. Establishing the pattern of ice nucleation and inhibition in these diverse insect orders will extend knowledge of overwintering physiology and the possible selective advantage of each strategy.

The significance of the proposed research is not only in understanding evolutionary "solutions" to extreme conditions and the limits of these mechanisms will significantly advance comparative physiology but also in advancing applied research in the areas of frost control. Knowledge of the mechanisms of biogenic ice formation will have trans-disciplinary impact, too. A key medical concern is the deleterious effects of increased solute concentration on tissue function. By imaging patterns of ice formation

in these diverse organisms, cryomedicine (Costanzo *et al.* 1995) will gain an understanding into the preservation of human tissues and organs with these already-developed evolutionary "solutions." Similarly, for physical scientists, my future research on tissues and compartments that inhibit, tolerate, and/or impede ice below the homogeneous ice nucleation temperature of water (-40°C) will showcase other biomimetic possibilities. Billions of dollars in lost revenue as well as material loss of thousands of hectares of plants such as citrus trees (Tiefenbacher *et al.* 2000) can be mitigated by visualizing and understanding biogenic ice formation in these organisms.

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Appendix 2: Limited overwintering physiology of *Hypnoidus bicolor*, *Phyllocnistis populiella*, *Camponotus herculeanus*, and an unidentified parasitic wasp.

Hypnoidus bicolor Eschscholtz, a click beetle

Hypnoidus bicolor (Coleoptera: Elateridae) is a beetle also known as "tundra" or more commonly a "click" beetle (Strong *et al.* 2002) due to its fully articulated prothorax that snaps and propels the beetle in a clicking habit (Arnett 1968). It has a palearctic distribution, common in the high and low arctic, but not found in Greenland (Danks 1981). While Brooks (1961) spells the scientific name *Hypolithus bicolor* and notes its

distribution from southern Alberta, Saskatchewan, and Manitoba (Churchill), Arnett (1968) and Danks (1981) note the various spellings of the genus in which *Hypnoidus* appears to have gained consensus. Strong *et al.* (2002) collected this species in higher elevations in Vermont, USA, at the Stowe Mountain Ski Resort (44 ° 32.6' N) between 1019 and 1097 meters. Brooks, citing Zacharuk 1958, states that "this species apparently reproduces pathogenically in the north and west as no males have been collected in the grey-soil [grey-soil not defined] forest areas" (p. 25). Strong *et al.* (2002) examined beetle community structure between forested areas and on ski trails as edge and disturbed sites. They found that *Hypnoidus* spp., including *H. bicolor*, were "in the ski trail" and not in the forested area.

Work on *Hypnoidus bicolor* was a group effort in which we documented the presence of thermal hysteresis activity, i.e., the presence of antifreeze proteins (Duman *et al.* 2004). Collection of insects was conducted at and north of the Toolik Field Station (Toolik, 68° 38'N), with one summer trip at Atigun Pass (June 2004); however, most effort focused on a Department of Transportation (DOT) site near the Sagavanirktok River called the Sag River DOT site (approximately 68° 45' N). This location is near active gravel pits that are being excavated for the Dalton ("Haul Road") Highway. The most fruitful collection of individuals was off a side road that formed a cul-de-sac. This site was overrun with vegetation (plants to shrubs) and many rocks. In general, this site could be described as disturbed, confirming the presences of this species in such sites as notes by Strong *et al.* (2002). To collect, we upended rock and found *H. bicolor*, among many species of spiders and at least one species of Collembola (Duman *et al.* 2004). We

also placed loggers to record above- and below-snow temperatures. In addition, individuals were placed above- and below- snow at the Toolik Field station near the "Winter Lab" in some years.

The earliest work on supercooling assessment was conducted at the Toolik Station, while in later years, individuals were brought back to UAF for testing. Assessment of both wet and dry supercooling was conducted by securing individuals to a thermocouple with Vaseline. This method continued until 23 September 2004, when I used smaller tubes that no longer required the use of Vaseline (the first non-Vaseline isothermex file is designated 092304c).

Representative mean supercooling (±SEM) points are shown in Figure A1 along with above- and below-snow temperatures at the Sag Site. In 2006, I used an Ibutton (Figure A2, only one channel) to record temperatures at the Toolik enclosure site. There is a seasonal increase in supercooling capacity from relatively high subzero supercooling temperatures of -7.7 ± 0.4 (N = 25) and water content of 1.3 ± 0.1mg · mg^{-1} dry mass (n = 21) in June 2004 and July 2005 (combined averages) to low supercooling ranges between -22 to -27 in December – March months (and over the years) while water content over this same overwintering period varied between 0.5 to 0.9 mg · mg^{-1} dry mass.

No individuals survived any freezing event. When we tested survival after retrieving overwintering containers at various times of the year and locations (Sag and Toolik sites), it appeared that temperatures in the -20 °C range was a limit, although individual supercooling points as low as -27 °C were recorded; however, due to many logger failures, especially early in the study between 2003-2005, we do not always have

temperature data. Also, abrasion from natural substrate–stones from the underside of overturned rocks–made it difficult to tell whether supercooling ability was compromised. We also had difficulty in transporting individuals back to Fairbanks. Since containers were not packed full with natural substrate and, therefore, stones could be easily jostled and potentially damage individuals. Early in the study, we placed collecting containers in un-insulated ice chests for the eight hour ride back to UAF. On some occasions, temperatures dipped below -35 °C, and we saw no survival in containers. At Toolik, placement of below-snow containers literally below the winter lab may also have been problematic. When traveling to Toolik in mid-winter, I saw at times either a thin layer of hard, wind-packed snow that could decrease the insulating cover or irregular drifts formed due to the building's pilings that resulted in snow cover that appeared much less "natural" than snow cover at the overwintering Sag site. One interesting note on temperature at the Toolik enclosure site was that during the 2006-2007 season, Toolik above-snow temperatures reached -47.8 °C, while at the same time, the below-snow temperature was near -19.8 °C. It was not until 22 March that below-snow temperature fell to its lowest of -20.9 °C. No above- or below- snow click beetle survived.

In a series of supercooling tests comparing dry vs. wet individuals, I found mixed results (Fig. A3) with some tests indicating that mean wet SCPs were lower than mean dry SCPs; however, this was not consistent. For instance, during four consecutive days of testing, tests on 15 and 16 March 2004 showed a trend toward lower wet SCPs, but on 17 and 18 March 2004, wet and dry SCPs appeared similar (Fig. A3). On 21 and 22

September 2003, wet SCPs were lower than dry SCPs. In contrast, 23 September 2004 indicated that wet SCPs were higher than dry SCPs.

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B. *Phyllocnistis populiella* Chambers, the aspen leaf miner

Phyllocnistis populiella is a lepidopertan (Gracillariidae). Population outbreaks of this species in Alaska have been reported from the 1950s to the present (Doak *et al.* 2007; Wagner *et al.* 2008). In 2005, the area of infestation of this herbivore of aspen (*Populus tremuloides*) was estimated to be greater than 600,000 acres (U.S. Forest Service 2005). Although the area of defoliation has decreased to 210,000 acres in 2007, this insect remains the most widespread pest in Alaska (U.S. Forest Service 2008) with its

telltale epidermal leaf mines found as far north as the south slopes of the Brooks Range to Talkeetna (U.S. Forest Service 2008).

Drs. P. Doak and D. Wagner (UAF/IAB) continue to examine the leaf miner in relation to quaking aspen ecology. Doak *et al.* (2007) found that extrafloral nectaries on short but not tall aspen ramets reduced leaf mining. Wagner *et al.* (2008) found that aspen growth, leaf longevity, and photosynthesis declined when mining damage occurred on the abaxial (underside) surface of leaves. When, experimentally, they restricted leaf miners to the adaxial (topside) surface, they found that photosynthesis was not significantly different than leaves without miners present. They concluded that while the leaf miners do not consume photosynthetic tissue of the mesophyll, miners most likely do consume tissue of guard cells that open and close stomata that are located on the abaxil surface. Water conductance, therefore, appears to be affected by the abaxil mines that then decrease photosynthesis.

I was approached by Drs. Doak and Wagner to co-mentor a high school student (see outreach). The project entailed determining the freeze-tolerance or freeze-avoiding status of the leaf miner as well as examining the seasonal changes in its supercooling capacity. Adult leaf miners overwinter in leaf litter at the base of trees (Doak *et al.* 2007; Wagner *et al.* 2008). To assess supercooling capacity, individuals were collected on the University of Alaska Fairbanks (UAF) campus and various locations surrounding Fairbanks. They were held outside (usually less than one hour after capture) until testing. Insects were placed on a pre-cooled watch-glass on ice in a standard refrigerator. Active individuals were allowed to crawl into the pre-cut (~ 20 mm) 0.4 mL microcentrifuge

plastic tubes, while inactive individuals were placed in tubes. A thermocouple (36 gauge) juncture was rested against the surface of an individual. Tubes were then placed inside a larger glass beaker that was placed in a cooling bath (Neslab). Once insects equilibrated to ~ 0 °C, bath temperature was reduced at 0.2 °C/min. The lowest body temperature recorded (Iso-Thermex) at the release of the latent heat of fusion, as evidenced by an exotherm, was taken to be the supercooling point (SCP).

The Brown-Forsythe homogeneity of variance test was performed. If variances were found to be heterogeneous ($P < 0.5$), a weighted mean was used for 1-Way ANOVA, with the post hoc Tukey-Kramer Adjustment for Multiple Comparison tests (SAS 9.1, SAS Institute, Inc). Means (±S.E.M.) were calculated from the supercooling points/run.

Representative mean supercooling (± SEM) points are shown in Figure B1. There is a seasonal increase in supercooling capacity from relatively high subzero supercooling temperatures of -16.7 ± 0.42 °C (n = 94) during August-September 2007 and April-June 2008 to low mean supercooling of -31.6 ± 0.45 °C (N = 100) for the remaining months.

On 13 August 2007, a supercooling test was run on individuals collected by D. Wagner from Ridge Point Drive (Fairbanks). One individual did not freeze when the bath had been taken down to approximately -20 °C; the rest (n = 14) froze (-16.4 ± 0.3 °C). This individual was the only one alive, indicating that leaf miners are freeze-avoiding insects. No individuals survived freezing (N = 14/14 on 13 August 2007, 8/8 on 31 October 2007, and 13/13 on 27 November 2007).

On 9 October 2007, individuals collected approximately 10 days prior and held in small containers outside were tested. Of the 6/7 showed movement when placed on the 4 °C watch glass. From approximately late September / October through the end of February, individuals did not show shins of movement when brought in from the outside and placed on the watch-glass. The earliest evidence of "recovery" from overwintering occurred on 29 February 2008. Pat Doak had collected individuals that morning, and I ran a supercooling test within an hour. On the watch-glass 6/15 either had legs moving or showed some amount of movement. By 27 March 2008, 10/14 displayed movement, and by 23 April 2008, 14/14 could be seen walking on the watch-glass.

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C. *Camponotus herculeanus* Linnaeus, carpenter ant

Camponotus herculeanus is an omnivorous boreal carpenter ant (Hymenoptera) in the family Formicidae (Heinze 1993). This species excavates wood for nesting sites (Nielson 1987). Besides this species, others formicids in the genus *Formica* and *Lusius* are known to be abundant in cool, boreal regions from Eurasia and North America (Fisher and Cover 2007), including Interior Alaska (Heinze 1993). The *C. herculeanus* individuals examined in this study (Ascension # UAM-2008.16-Sform-Ento) have been regularly observed but not collected and tested until September 2008. Individuals were collected from approximately seven trees at 12 mile Cache Creek (64° 54' N) outside of Fairbanks, Alaska.

In September, while tearing apart standing or downed dead trees or stumps, ants could be found moving en mass, most likely due to a nest at or below ground of the stumps. Large queens were observed along with two size classes of workers. Fisher and Cover (2007) note that worker castes can be di- or poly-morphic.

During the September collecting, many queens were found within one tree and were mixed with both worker caste sizes, so it was not possible to tell whether a stump served as a single nest that had many queens. Eidmann (1943) found the same overwintering collection of size classes and queens and even noted that larvae could also be found (as paraphrased in Henize 1993). I did not examine overwintering sites well enough to conclude where larvae or eggs could be found at this time in preparation for overwintering. In October, after freeze-up, ants were found in horizontal logs of various tree species. After peeling away bark, small circular cells that contained both sizes

workers but usually only one queen could be found. Since these cells were closest to ambient conditions, temperatures most likely faced by these ants could be similar to temperatures recorded by Bennett *et al.* (2005), where they note poorly insulated horizontal log temperatures measured under the bark, Fig. 1A). On two occasions, the interior of logs were fully dismantled. These logs were located on the ground and would have better insulated both worker classes were found in restricted areas but in less well-formed cells and with more than one queen.

Heinze (1993) noted that many species of boreal ants have a system known as social budding whereby more than one queen overwinters in a nest and "spontaneous fractioning of colonies (budding)" takes place in the spring. Nielson (1987) stated that Interior Alaska sexuals are produced late in summer and overwinter, and Hölldobler and Wilson 1990 also noted generally that *C. herculeanus* male and females overwinter in maternal nests. Mating flights and dispersal take place in June, and individuals can be carried aloft (Nielson 1987). The most northern collection of this species in Alaska occurred at Sukakpak Mountain (approximately 67 °N), north of Wiseman, Alaska (Nielson 1987).

Ant overwintering research is not extensive, and almost nothing has been conducted in Interior Alaska. Heinze (1993), citing Leyrikh 1989, found that overwintering species *Leptothorax acervorum* (subfamily Myrmicinae) from Siberia possessed anti-freeze polyols and could survive -40 °C. This species is also known to be in Interior Alaska (Heinze 1993). Since ants were plentiful in September and October 2008, I decided to test overwintering status to determine whether they were freeze

tolerant, freeze avoiding, and even simultaneous tolerance and avoidance since these ants have a pronounced "wasp waisted" connection between the thorax and abdomen. Also, since hymenoptera have not been found to possess antifreeze protein (Duman *et al.* 2004), this seemed to be a good candidate to test for thermal hysteresis activity (THA).

Supercooling testing was conducted, and the Brown-Forsythe homogeneity of variance test indicated homogeneous variance ($P > 0.2$) for water content and supercooling points (Fig. C1). A 1-Way ANOVA, with the post hoc Tukey-Kramer Adjustment for Multiple Comparison tests was used (SAS 9.1, SAS Institute, Inc). This species displayed two freezing events per individual, termed supercooling point 1 (SCP1) and supercooling point 2 (SCP2). Representative mean supercooling points ($\pm$ SEM) and water contents are shown in Table C1, while Figure C1 displays the spread of SCP1, SCP2, and WC over the testing periods and under treatments. Survival after the first freezing events was conducted on 8 October 2008 by holding ants at -15 °C for 24 hours. After returning to 0 °C, ants were placed on ice for 24 h and then transferred to a refrigerator between 2 to 4 °C in 100 % RH. These were checked every day. By the time I returned in October to collect more ants, snow had fallen, and ants were not as easy to find; therefore, survival of the second freezing event was not conducted.

For thermal hysteresis analysis, a 26 gauge needle was used to pierce the various hard body parts, and a pulled glass micropipette was used to wick-up a small volume of hemolymph (≤ 0.25 µl). A micrometer syringe then delivered between 25 and 100 nl of hemolymph into heavy mineral oil located in the sample well of a nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand). The sample was frozen by cooling to -40

°C and then warmed until a single ice crystal was attained. The melting and freezing points of the ice crystal, as well as its growth morphology, were determined at 300x magnification.

Thermal hysteresis results (Table C2) indicate very little to no hysteresis, and the ice crystal shape (round) indicates that these individuals do not possess antifreeze proteins; however, the melting points of hemolymph samples from the thorax and abdomen (~ -2 to -5 °C) indicate that polyols are present (Table C2). Only one sample from the head was measured, but its low melting point indicates that substantial concentration of polyols (approximately 5.3 Osmol.) are present. Although only one sample from the head was tested, it may be speculated that the lower supercooling point SCP2 is associated with the greater concentration of polyols in the head.

Raw data for both supercooling points (1 and 2) and WCs are provided for convenience in Appendix 2.

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D. Parasitic Wasp. An unknown parasitic wasp (Ascension # UAM100023847)

According to Dr. D. Sikes (UAF/IAB), parasitic wasp species number in the thousands and are undergoing a major taxonomic re-evaluation; consequently, no one has been willing to identify to species. Our best attempt at having it identified was by photos sent to Dr. J. F. Triana, who stated in an email to Sikes on 8 April 2009:

> . . . belongs to the subfamily Ichneumoninae. [. . .] Andy Bennett (a CNC specialist on Ichneumonidae) and I just looked at your photos closely and we think it COULD be the genus Ichneumon-which have almost 700 described species, over 150 within the Nearctic . . . We cannot be sure of the genus though . . . Andy suggested that you should contact David Wahl at the AEI in Gainesville. He is the best expert on that group at present and would be able to provide you with a more accurate and complete ID.

The inclusion of this quotation is used to illustrate the fundamental level at which much of the insect overwintering work begins (at least in Alaska) and the importance of taxonomy. Even among professionals, the identity of insects can be problematic, and the parasitic wasp serves as an example (in another instance, initial work was begun on centipedes, which, it turns out, could only be identified by a taxonomist from Italy; furthermore, he noted that the sample contained three species).

Forty-one individual parasitic wasps were collected in early October 2009 at 12 mile Cache Creek (64° 54' N) outside of Fairbanks, Alaska. All came from one horizontal log that was about one meter off the ground. The wasps appeared to be in a

loosely formed cell. Since parasitic wasps have been known to be pathogenic (as noted by Dr. J. Avise during an IAB seminar entitled: Clonality: Genetics, Ecology and Evolution of Sexual Abstinence on 7 May 2009), this single collection of wasps from one tree could potentially be clones and, therefore, may not be independent and constitute an N =1, rather than 40 (one individual was crushed in the container). Despite this caution, overwintering status, mean supercooling points and water content, and thermal hysteresis analysis was conducted under the assumption of independence. These individuals also displayed a very narrow "wasp waisted" connection between thorax and abdomen and, therefore, a candidate for dual supercooling points. At the same time that supercooling testing was conducted, survival testing was also done to see if individuals were freeze tolerant (see method above). Finally, I also tested for thermal hysteresis activity with the nanoliter osmomenter (see method above).

Once collected, individuals were stored at the UAF/IAB insect enclosure on the ground. With only 40 individuals, testing was conducted twice. On 16 February and on 27 April 2009, supercooling tests were conducted, resulting in only one freezing event, despite the bath being taken down to -70 °C. Supercooling points and water contents are shown in Table D1. Based on the survival data, the wasp is freeze tolerant, having an average supercooling point of -9 °C (combined average, Table D1). Although data are few, it appears that over the winter, WC increases, SCPs decrease, and the proportion that survive after freezes decreases. The prediction of dual supercooling events was not confirmed.

Thermal hysteresis values as well as qualitative assessment of ice crystal shape are shown in Table D2. The value of 0.2 and greater in thermal hysteresis is consistent with the presence of antifreeze proteins in freeze-tolerant insects. In addition, crystal shape associated with these values would also indicate antifreeze proteins. This may be the first case of antifreeze proteins in hymenoptera.

Figures (Appendix 2)

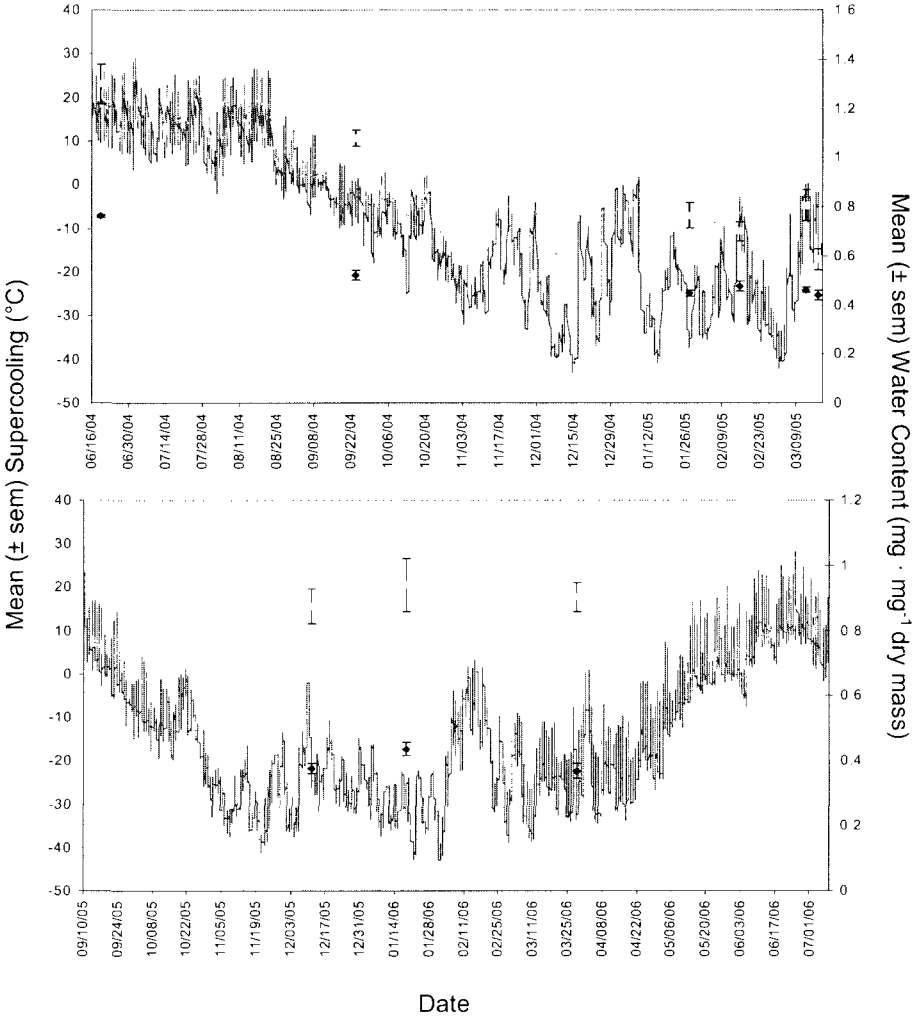

Figure A.1. Above- and below- snow temperatures (blue and grey lines, respectively) near the Sagavanirktok "Sag" River site 2004–2006. Gold squares are water content and black diamonds are supercooling points for *Hypnoidus bicolor*.

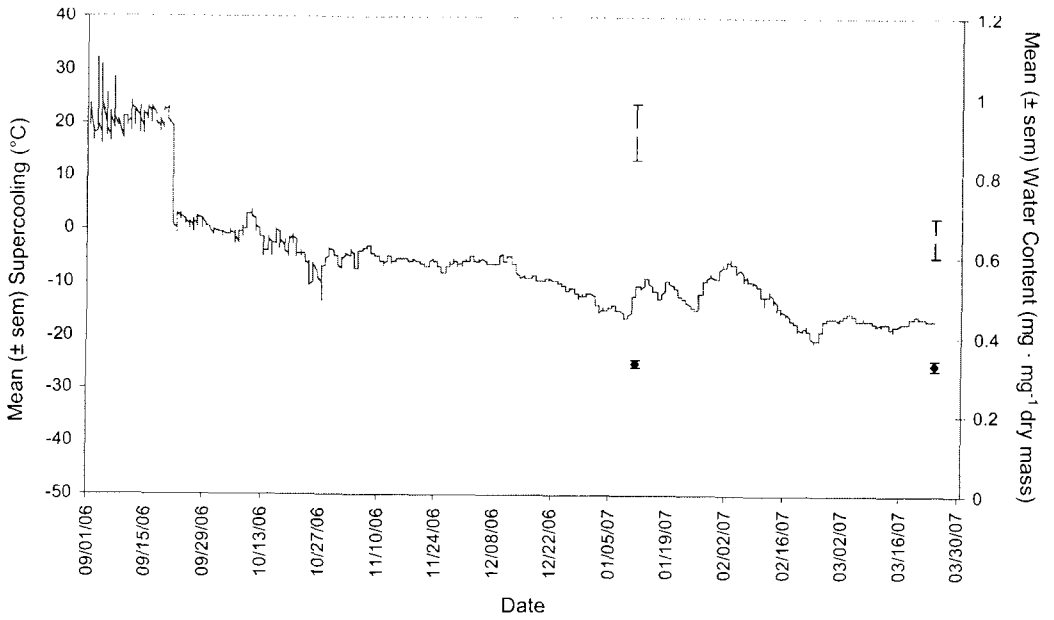

Figure A.2. Below-snow temperatures (blue line) from the Toolik enclosure site 2006–2007 for *Hypnoidus bicolor*. Gold squares are water content and black diamonds are supercooling points (SCPs).

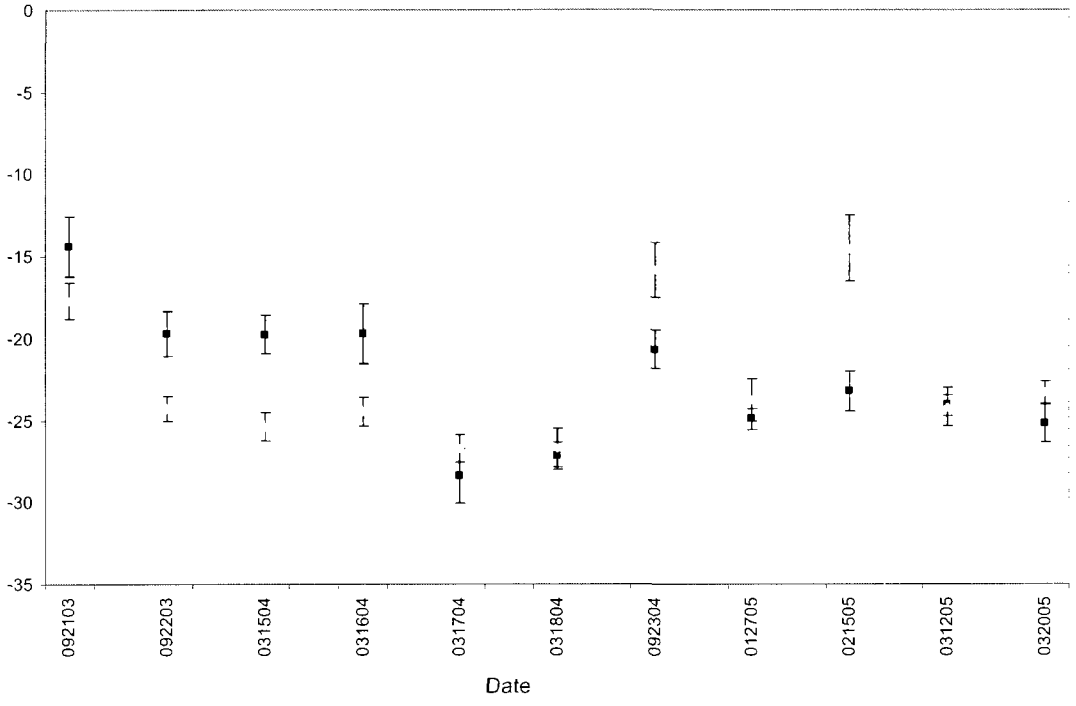

Figure A.3. *Hypnoidus bicolor* dry (black squares) vs. wet (gold diamonds) mean (± sem) supercooling points. Note that on several occasions wet SCPs are lower than dry SCPs.

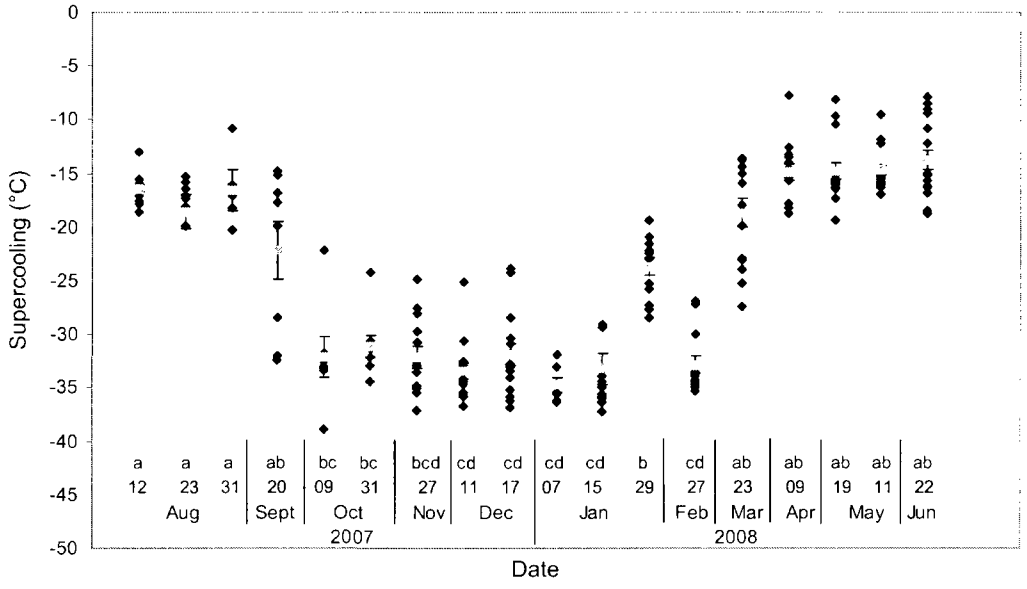

Figure A.4. Aspen leaf miner (*Phyllocnistis populiella*) supercooling points. Mean (±sem) SCPs are shown as gold squares and individual supercooling points are shown as blue diamonds. Different letters above dates indicate significant differences ($P < 0.05$) with the Tukey-Kramer adjustment for multiple comparisons.

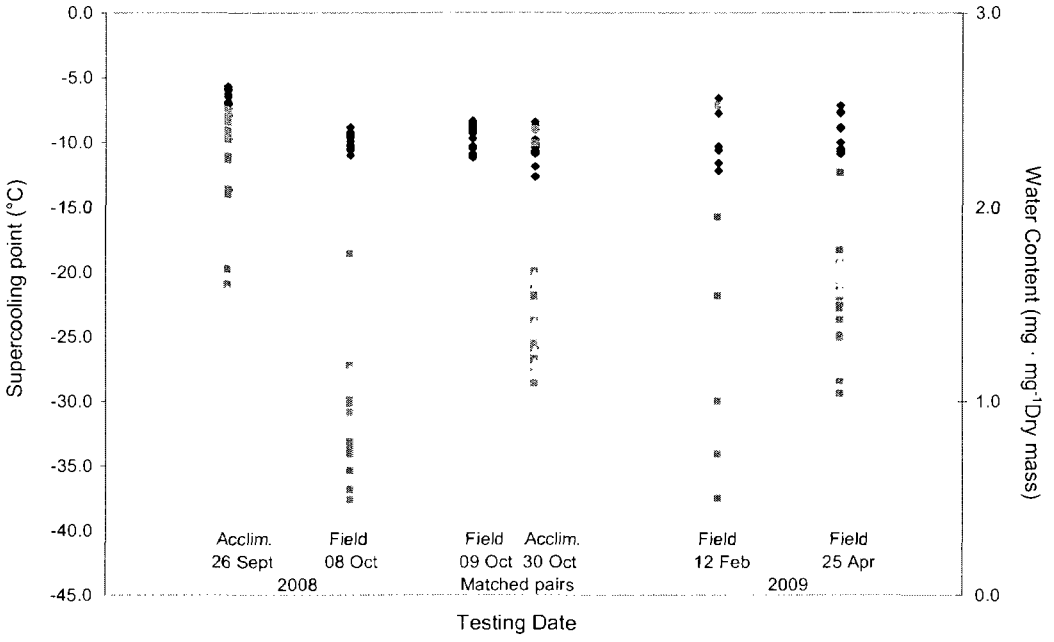

Figure A.5. The range of *Camponotus herculeanus* supercooling points (°C) (SCP1= blue diamonds, SCP2 = pink squares) and water contents (gold triangles) by testing date.

Tables (Appendix 2)

Table A.1. Mean (±sem) supercooling points (°C) for the two supercooling events, SCP1 and SCP2, and water content (percent) for *Camponotus herculeanus*.

Date	mean SCP1	sem	mean SCP2	sem	mean % WC	sem
09/26/08	-7.3[a]	0.5	-12.1[a]	1.2	54.9[a]	1.4
10/08/08	-9.8[b]	0.2	-31.1[ab]	1.2	NA	NA
10/09/08	-10.0[b]	0.2	-9.8*	0.2	NA	NA
10/30/08	-11.4[b]	1.1	-22.5[a]	1.7	59.1[b]	0.9
02/12/09	-9.6[a]	0.7	-24.4[ab]	4.7	53.7[a]	0.9
04/25/09	-9.1[a]	0.5	-22.5[a]	1.3	62.1[b]	0.9

Superscript letters indicate significant difference in means ($P < 0.05$) within category using Tukey-Kramer adjustment for multiple comparisons.

Table A.2. Thermal hysteresis (th) analysis calculated from melting points (mp) and freezing points (fp) from the hemolymph of *Camponotus herculeanus* queens on 8 October 2008. All ice crystals were formed in the nanoliter osmometer and appeared round.

mp	fp	th	body part
-2.2	-2.24	0.04	ab
-5.08	-5.18	0.1	th
-4.63	-4.71	0.08	th
-2.72	-2.77	0.05	th
-4.66	-4.79	0.13	th
-2.77	-2.83	0.06	th
-2.79	-2.84	0.05	th
-4.24	-4.37	0.13	th
-9.96	-10.15	0.19	h

Abbreviations: ab = abdomen, th = thorax, and h = head

Table A.3. Mean supercooling points (SCP °C) and water contents (mg · mg^{-1} dry mass) are shown for two testing dates for an unidentified species of parasitic wasp.

Date	Mean SCP[1]	sem	Mean WC[2]	sem	Survival
02/15/09	-9.9[a]	0.34	1.1[a]	0.03	8/8
04/27/09	-8.1[b]	0.18	1.5[b]	0.03	8/14

Superscript letters indicate significant difference in means ($P < 0.05$) within category.

[1] SCP: T-test, equal variance, t = 2.18, df =28, $P < 0.03$

[2] WC: Wilcoxon S = 37.5, $P = 0.001$

Table A.4. Thermal hysteresis (th) analysis on parasitic wasp. Thermal hysteresis was calculated from melting points (mp) and freezing points (fp) from the hemolymph of wasps bled on 15 February 2009. In addition, ice crystal character is described as round (characteristic of little to no antifreeze protein), slight hexagonal shape (characteristic of the presence of some small quantity of antifreeze protein), and strong hexagonal shape (characteristic of the presence of a larger quantity of antifreeze protein).

mp	fp	th	Ice Crystal Character
-8.44	-8.95	0.51	hexagonal, strong
-7.32	-7.49	0.17	round
-8.48	-8.72	0.24	hexagonal, strong
-6.23	-6.28	0.05	round
-6.13	-6.31	0.18	round
-5.37	-5.46	0.09	round
-7.01	-7.22	0.21	hexagonal, slight
-7.83	-7.98	0.15	hexagonal, slight

Appendix 3. Table of raw data collected for *Camponotus herculeanus*.

Date	scp1 (°C)	scp2 (°C)	Fresh Mass (mg)	Dry Mass (mg)	Percent Water	Water Content ($mg \cdot mg^{-1}$ Dry Mass)	Survive (1= yes, 2 = no, "." = NA)
09/26/08	-5.7	-9.8	42.20	17.8	57.9	1.4	.
	-7.0	-21.0	70.70	34.9	50.7	1.0	.
	-9.7	-11.4	40.80	17.8	56.4	1.3	.
	-6.9	-8.4	58.30	26.0	55.4	1.2	.
	-13.6	.	28.60	11.1	61.2	1.6	.
	-7.4	-19.8	72.70	38.2	47.5	0.9	.
	-6.3	-9.1	43.10	17.1	60.3	1.5	.
	-7.9	-19.8	78.80	37.8	52.0	1.1	.
	-5.7	-7.9	67.40	32.2	52.3	1.1	.
	-6.5	-11.4	54.60	24.5	55.1	1.2	.
	.	.	29.90	.	.	.	1
	-5.9	-11.1	74.80	.	.	.	0
	-7.1	-13.6	62.40	.	.	.	0
	.	.	69.80	.	.	.	1
	.	.	73.50	.	.	.	1
	-7.8	-14.0	73.50	.	.	.	0
	-6.9	-7.5	64.80	.	.	.	0
	-6.0	-9.2	63.30	.	.	.	0
	.	.	81.40	.	.	.	1
	-6.0	-7.8	68.90	.	.	.	1
10/08/08	-9.4	-34.1	16.07	6.8	57.8	1.4	.
	-9.9	-27.3	4.91	1.7	66.4	2.0	.
	-10.5	-33.5	18.76	8.2	56.5	1.3	.
	-9.6	-33.3	5.35	2.1	60.2	1.5	.
	-10.3	-33.1	15.21	6.7	55.8	1.3	.
	-9.5	-30.0	9.69	4.1	57.7	1.4	.
	-9.2	-26.0	6.15	2.4	61.6	1.6	.
	-11.0	-30.2	13.08	5.7	56.8	1.3	.
	-10.6	-35.4	56.95	22.1	61.2	1.6	.
	-10.2	-30.9	62.86	29.1	53.7	1.2	.
	-9.5	-36.9	111.24	48.8	56.2	1.3	.
	-8.8	-29.9	11.58	5.1	56.0	1.3	.
	-9.3	-30.1	10.86	4.9	55.2	1.2	.
	-11.0	-37.6	74.24	34.3	53.8	1.2	.
	-9.5	-30.0	78.34	37.6	52.0	1.1	.

	-9.2	-18.6	6.90	2.7	60.9	1.6	.
10/09/08	-9.0	.	63.97	.	.	.	1
	-8.8	.	39.40	.	.	.	1
	-11.1	.	22.50	.	.	.	1
	-8.5	.	91.02	.	.	.	1
	-9.3	.	5.92	.	.	.	1
	-8.3	.	81.27	.	.	.	*
	-10.3	.	13.32	.	.	.	1
	-8.7	.	10.62	.	.	.	1
	-9.1	.	5.32	.	.	.	1
	-10.4	.	9.24	.	.	.	1
	-10.9	.	15.33	.	.	.	1
	-11.1	.	23.99	.	.	.	1
	-10.5	.	11.53	.	.	.	1
	-11.2	.	10.51	.	.	.	1
	-9.7	.	9.92	.	.	.	1
	-10.4	.	15.94	.	.	.	1
10/30/08	-9.0	-26.8	65.80	30.0	54.5	1.2	.
	-25.9	-25.5	39.43	17.5	55.7	1.3	.
	-12.6	-28.4	23.03	9.2	60.0	1.5	.
	-8.7	-10.1	90.99	42.3	53.6	1.2	.
	-10.1	-10.1	6.47	2.3	65.1	1.9	.
	-8.4	-9.0	76.52	35.8	53.3	1.1	.
	-10.4	-28.6	13.18	5.5	58.1	1.4	.
	-10.1	-26.8	11.36	4.3	62.2	1.6	.
	-9.8	-20.0	5.55	2.0	63.8	1.8	.
	-10.8	-26.1	9.62	3.5	64.0	1.8	.
	-10.9	-27.1	16.16	6.3	61.3	1.6	.
	-11.9	-23.8	24.81	9.4	62.3	1.7	.
	-10.8	-27.0	12.18	5.2	57.3	1.3	.
	-10.7	-27.5	10.18	4.4	57.3	1.3	.
	.	-22.0	10.11	4.2	58.1	1.4	.
	-10.3	-21.4	15.84	6.6	58.5	1.4	.
02/12/09	-12.2	.	52.88	23.7	55.2	1.2	.
	-11.6	-30.0	16.30	7.6	53.6	1.2	.
	-10.3	-37.5	11.69	5.5	53.0	1.1	.
	-6.6	-7.2	55.76	26.1	53.3	1.1	.
	-7.7	.	57.60	27.8	51.8	1.1	.
	-7.7	-15.8	68.63	35.1	48.9	1.0	.
	-10.3	-34.1	17.11	7.4	56.9	1.3	.
	-10.6	-21.9	10.54	4.6	56.8	1.3	.
04/25/09	-7.6	-24.9	7.26	2.5	66.0	1.9	.

	-7.7	-12.4	13.07	4.5	65.8	1.9	.
	.	-18.3	12.22	4.7	61.8	1.6	.
	-10.5	-21.2	12.66	4.7	62.8	1.7	.
	-8.9	-28.4	6.11	2.3	63.0	1.7	.
	-8.8	-23.7	11.43	4.2	63.0	1.7	.
	.	-29.4	3.69	1.4	61.0	1.6	.
	-10.9	-22.6	16.61	6.1	63.2	1.7	.
	-7.2	-25.1	3.93	1.5	62.3	1.7	.
	-10.7	-22.0	28.71	10.9	62.1	1.6	.
	.	-22.8	64.84	30.2	53.4	1.1	.
	-10.0	-19.3	10.98	4.3	61.2	1.6	.